DOCTORAL THESIS

Transcriptional analysis caveolae-related transcripts in the ischaemic-intolerant ageing mouse heart

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Award date:
2016

Transcriptional analysis of caveolae-related transcripts in the ischaemic-intolerant ageing mouse heart

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Submitted in total fulfilment of the requirements of the degree of Doctor of Philosophy by Research

July 2016

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ABSTRACT

Ischaemic heart disease (IHD) is the most prevalent of the cardiovascular diseases and the largest single leading cause of death in Australia and other Western nations. Currently, there are no effective treatments that salvage the ischaemic myocardium as well as limiting the deleterious effects of reperfusion. Cardioprotection is a promising novel therapeutic for the treatment of IHD as cardioprotective interventions have been shown to limit both ischaemic and reperfusion (I-R) injury. However, its clinical translation has been limited due to its poor efficacy in the aged heart. Cardioprotective interventions such as ischaemic preconditioning as well as numerous pharmacological approaches are reliant on the presence of caveolae and present a novel therapeutic target as enhanced expression of caveolae has been shown to be protective against variety of stressors such as I-R injury in the aged heart. The unique morphology of caveolae arise from the expression of its coat proteins belonging to those of caveolin, cavin and Popdc family of proteins with differential expression of these protein shown to result in tissue-specific formation of caveolae.

Whilst the age-related expression of Caveolin-3 (Cav3) has been investigated in the hearts, the expression of other caveolae-related transcripts is largely uncharactiersed. Their involvement may help partially explain age-related changes in cardioprotection. To show this, we sought to characterise age-dependant expression of caveolins, cavins and Popdcs in the ageing normoxic and post-ischaemic Langendorff model using transcriptional and immunoblotting analysis. Most murine ageing studies have focused on the aged/senescent phenotype (>74-week old), which show the efficacy of cardioprotective interventions as well as I-R tolerance is lost when compared to young (8-16 week old) counterparts.

Here we show that the loss of ischaemic tolerance is evident in male middle-aged mouse hearts (48-week old) as shown by enhanced LDH release and post-ischaemic contractile dysfunction. Aged-related down-regulation of crucial caveolae transcripts *Cav3*, *Cavin1* and *Popdc1* was observed in middle-aged male hearts, which may partially explain the loss of protection in these hearts. We also characterised caveolar gene expression in the ageing female myocardium. As previous experimental and epidemiological studies in the ageing female hearts suggests they are more ischaemic tolerant. However, we did not observe this cardioprotective phenotype in ageing female hearts when compared to male counterparts.

Coincident with the loss of cardioprotection in female hearts, the expression of *Cav3* and *Cavin1* showed similar patterns to male counterparts, although the relation between mRNA and protein was less consistent in females.

We sought to further investigate the involvement of caveolae (specifically *Cav3*) in ischaemic tolerance by employing an RNAi cell culture model. Whilst this transfection rate was comparable to that of literature using chemical transfection methods, the degree of knockdown obtained with such rates is not associated with reliable repression of *Cav3* mRNA. Thus, pharmacological disruption (MβCD) of caveolae was employed to show caveolae involvement in modifying ischaemic tolerance. MβCD-treatment following simulated ischaemia-reperfusion resulted in increased cell death when compared to non-treated simulated ischaemia-reperfused HL-1 cardiac cells.

Recently, a novel type of small RNA molecules termed microRNAs (miRNAs) have emerged as important regulators of ageing, ischaemia-reperfusion injury and ischaemic conditioning. We sought to investigate the expression of several key miRNAs in the ageing male hearts, namely those implicated to have a role in ageing and ischaemia-reperfusion. Of the miRNAs associated with ageing, only *miR-378* was differentially expressed in both the normoxic and post-ischaemic ageing hearts. Bioinformatic prediction was used to identify miRNA candidates targeting *Cav3* and *Cavin1* transcripts. No inverse-relationship was observed for miRNAs predicted to target *Cav3* and *Cavin1* in the ageing heart.

Increasing evidence suggests that caveolae and their coat proteins are crucial contributors to the plasma membrane response. This is shown by the accumulation of caveolae-like vesicles near the sites of injury. Of the plasma membrane repair transcripts, only *Anxa5* showed significant down-regulation with ageing in the normoxic hearts. There was also marked induction of proteases *Trim54* and *Calpain3,* these may be responsible for the reduction of recovery observed in the young-adult and middle-aged hearts although this remains to be shown at the protein level.

In conclusion, here we show significant age-related reductions in I-R tolerance in both male and female hearts, evident by 32-weeks of age (prior to 'middle-age'), with middle aged hearts exhibiting marked exaggeration of oncosis and contractile dysfunction. Age-dependent impairment of stress-tolerance was associated with significant baseline reductions in cardiac

transcript for all three caveolins in male but not female tissue, which was conserved for *Cav3* in post-ischaemic tissue. These data reveal age-dependent repression of transcript and protein for critical caveolar proteins (*Cav3*, *Cavin1* and *Popdc1)* that are more pronounced in male hearts compared to female counterparts. Through mechanisms yet identified, age appears to suppress transcription and expression of multiple caveolar proteins relevant to cardiac stress responses, although female hearts exhibit lesser changes than males. Taken together, age-related down-regulation of caveolae which co-localise several crucial cardioprotective receptors and caveolar coat proteins which help target plasma membrane repair proteins to sites of injury may help partially explain reduced ischaemic tolerance in these hearts as well as reduced responsiveness to therapeutic intervention. Indeed, restoration of caveolae in the aged heart is associated with increased recovery and mechanisms leading to restoration of caveolae in the aged hearts have been suggested to be therapeutic for IHD.

ACKNOWLEDGEMENTS

I would like to thank Associate Professor Kevin Ashton for accepting me as a PhD candidate thus providing me with this fantastic opportunity. I would like to thank our Associate Professor Jason Peart and Dr. Louise See Hoe for their generous support with the immunoblotting. I would like to thank the laboratory staffs (John, Marion, Jan and Ester) who have been continually helpful in various areas of teaching as well as research. I would like to thank the family Kaya for the Sunday barbeques as well as their hospitality. Thank you to my Canadian friends (Bjorn, Rami and Sarvesh) for their hospitality during the last months of my PhD and understanding that there is no rest for the weary. I would like to thank my family for their unflinching support.

"*Those who seek gold dig up much earth but find little*"

-Heraclitus

DECLARATION

This thesis is submitted to Bond University in fulfilment of the requirements of the degree of Doctor of Philosophy by Research.

This thesis represents my own original work towards this research degree and contains no material which has been previously submitted for a degree or diploma at this University or any other institution, except where due acknowledgement is made.

Can Justin Kiessling

July 2016

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ABBREVIATIONS

7-AAD	7-Aminoactinomycin D
ACTB	Beta actin
AMI	Acute Myocardial Infarction
ANP	Atrial Natriuretic Peptide
AR	Adenosine Receptor
B2	Bradykinin receptor 2
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
Capn1	Calpain 1
Capn2	Calpain 2
Capn3	Calpain 3
Cav1	Caveolin-1
Cav2	Caveolin-2
Cav3	Caveolin-3
CCPA	2-Chloro-N6-cyclopentyladenosine
Anxa1	Annexin1
Anxa5	Annexin5
Anxa6	Annexin6
cDNA	Complementary DNA
CVD	Cardiovascular Disease
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine
DNA	Deoxyribonucleic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
eNOS	Endothelial Nitric Oxide Synthase

Erk1/2	Extracellular Signal Regulated Kinase 1/2
GFP	Green Fluorescent Protein
GPCR	G-protein Coupled Receptor
GSK3β	Glycogen Synthase Kinase 3 Beta
GTP	Guanosine Triphosphate
Gαi	G-alpha Inhibitory
Gαs	G-alpha Stimulatory
I-R	Ischaemia-Reperfusion
IHD	Ischaemic Heart Disease
IPC	Ischaemic Preconditioning
IPOST	Ischaemic Postconditioning
JAK/STAT	Janus kinase/Signal Transducer and Activator of Transcription
kD	KiloDalton
MAPK	Mitogen Activated Protein Kinase
miRNA	MicroRNA
mitoK$_{ATP}$	Mitochondrial KATP Channel
MMP	Matrix Metalloproteinase
mPTP	Mitochondrial Permeability Transition Pore
mRNA	Messenger RNA
MβCD	Methyl-beta-Cyclodextrin
PBS	Phosphate Buffered Saline
PCI	Percutaneous Coronary Intervention
Pgk1	Phosphoglycerate Kinase
PI3K	Phosphoinositide 3-Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C

POPDC	Popeye Domain Containing
Ppia	Peptidylprolyl Isomerase A
RISC	RNA Induced Silencing Complexes
RNA	Ribonucleic Acid
RNAi	RNA Interference
ROS	Reactive Oxygen Species
Rpl13a	Ribosomal Protein L13a
RT-qPCR	Reverse Transcription-quantitative Polymerase Chain Reaction
RTK	Receptor Tyrosine Kinase
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
sI/R	Simulated Ischaemia-Reperfusion
siRNA	Small Interfering RNA
Smpd1	Sphingomyelin Phosphodiesterase 1
Smpd2	Sphingomyelin Phosphodiesterase 2
TGFα	Transforming Growth Factor-α
Trim	Tripartite motif family
VDAC	Voltage-dependent anion channel

1. Chapter One

General Introduction

1.1 Cardiovascular Disease

Cardiovascular disease (CVD) is the leading cause of death and was responsible for over 17 million (or 30%) of non-communicable worldwide deaths in 2008 (WHO 2011). In 2013, CVD in Australia was responsible for claiming the lives of approximately 43,950 Australians, or 30% of all annual deaths (Heart Foundation 2012). CVD is a broad category of diseases including ischaemic heart disease (IHD), cerebrovascular disease (including stroke), congestive heart failure, rheumatic heart disease and hypertension. IHD (a.k.a. coronary heart disease) is the most prevalent CVD contributing to 44% of CVD cases. As shown in Figure 1.1B, IHD is the largest single leading cause of death in Australia with over 19,000 reported deaths (or 15%) in 2011 (ABS 2011; Heart Foundation 2012). The incidence of IHD increases with age, affecting 29% of those aged 75 years and over and 47% of those aged 85 years and over. In addition, men more likely to have a heart attack and die as a result of IHD when compared to females (ABS 2014; AIHW 2015). As shown in Figure 1.1A, IHD was the leading cause of death for males and females in Australia in 2013. Several risk factors contribute to development of IHD including smoking, diabetes, high cholesterol and triglycerides, unhealthy diets and sedentary life styles (Anderson et al. 1991).

IHD occurs when the heart is deprived of oxygen and nutrients received from the coronary arteries. These arteries can be blocked through gradual plaque formation often due to atherosclerosis. The formation of this plaque involves a complex interaction of immune cells, changes in the extracellular matrix around the intima, and deposition of cholesterol particles which enlarge over time and can be accelerated by several risk factors of IHD (see review Libby, Ridker & Hannson 2011). The occlusion can rupture causing a complete clot and blockage of the artery resulting in acute myocardial infarction (AMI) of the downstream tissue (Figure 1.2). Without immediate intervention, development of myocardial necrosis develops due to inadequate perfusion of the distal tissue which can be observed as the “wavefront necrosis” phenomenon. Current AMI treatment guidelines emphasise percutaneous coronary intervention to re-establish reperfusion to myocardium with fibrinolytic and other anticoagulant therapies used at pre-hospital scenarios (O’Gara et al. 2013). Although reperfusion has been shown to be beneficial in salvaging myocardium, reperfusion has been widely recognised as to contribute as much as 50% to the final infarct size (Braunwald & Kloner 1985).

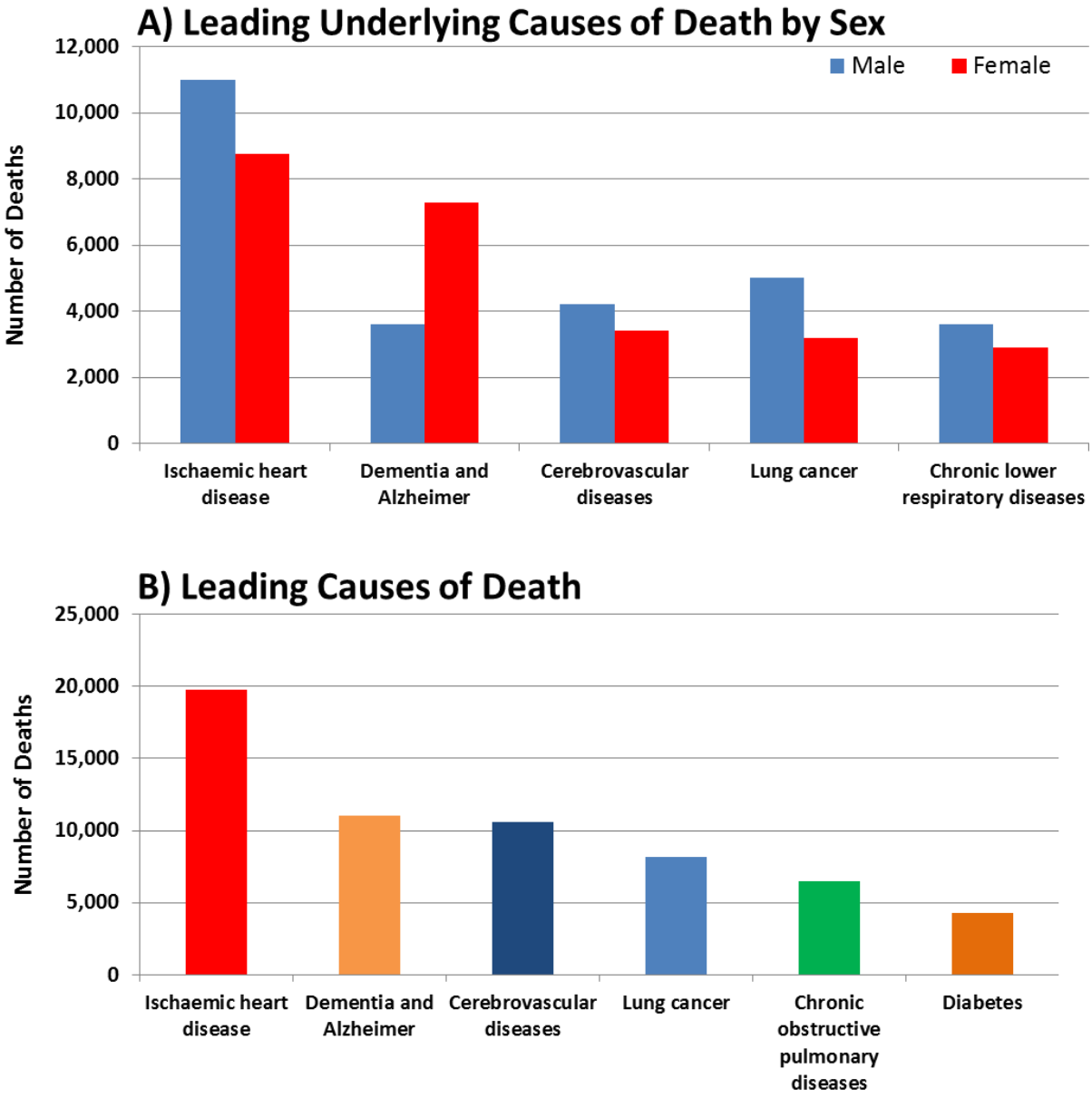


Figure 1.1 A) In Australia 2012 & 2013, IHD was the leading cause of death (≈19,000) in both the male and female population. **B)** IHD outranks cerebrovascular, mental illnesses, lung cancer, chronic obstructive pulmonary diseases and diabetes (Compiled from ABS 2012; AIHW 2013).

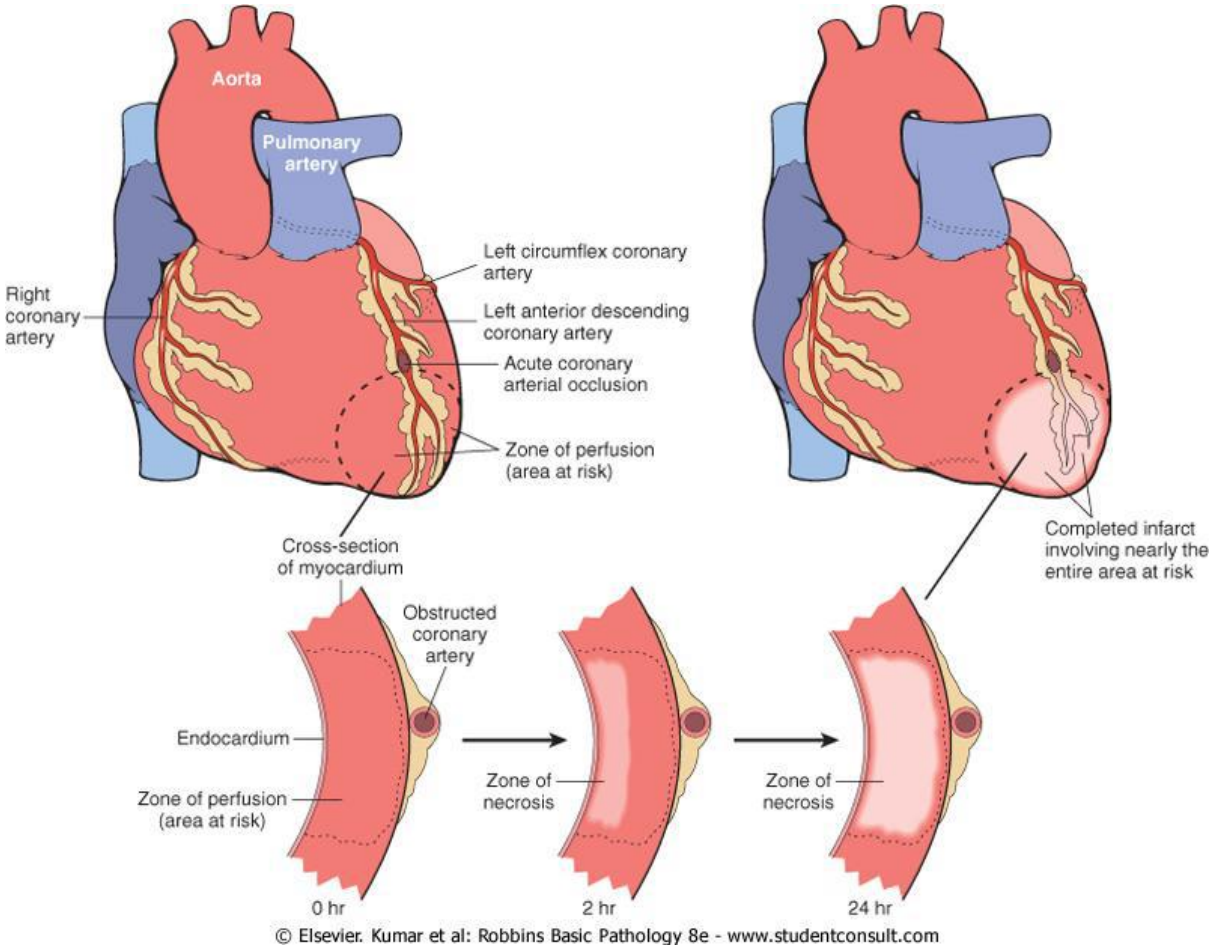


Figure 1.2 Occlusion of a major coronary artery results in ischaemia and progressive necrotic cell death of distal myocardial tissue. (Taken from Kumar et al. 2007).

1.2 Ischaemia-Reperfusion

Ischaemia results from insufficient blood flow providing inadequate oxygenation and nutrients to a tissue (Klabunde 2010). The most common cause of ischaemia in the heart is the narrowing or blockage of a major coronary vessel (i.e. clotting, atherosclerosis) which results in substrate deprivation of the downstream tissue. As shown in Figure 1.2, coronary occlusion induces ischaemia at the distal perfused tissue with wavefront of necrosis extending from the endocardium to the epicardium which can be observed within a 3-4 hour period. The final infarct size is dependent on the duration and severity of the ischaemic period and availability of arterial collateral supply with irreversible cell injury beginning around 30-60 minutes in the endocardium (Buja 2005; Reimer & Jennings 1979). Necrosis, apoptosis and oncosis have all been shown to contribute to cell death in the ischaemic zones. However, there has been some debate about the extent of apoptosis occurring in the ischaemic myocardium and whether oncosis or apoptosis is the major mechanism of cell death. These differences can be mainly attributed to variations observed in human versus animal human studies (Elsässer et al. 2000).

Ischaemia leads to a dramatic reduction in mitochondrial ATP production which has several consequences on the cell. Oxygen deprivation results in a metabolic switch from oxidative phosphorylation to anaerobic glycolysis, which cumulates in intracellular acidosis (Williamson et al. 1976). Intracellular accumulation of protons activates the Na^+/H^+ ion exchanger with concomitant loss of $3Na^+/2K^+$ ATPase resulting in intracellular Na^+ overload (Karmazyn et al. 1996). Persistent ischaemia leads to swelling of the lysosomes, mitochondria and the cell due to a loss of Na^+ pumps; these are typical indicators of irreversible cell injury. Build-up of Na^+ ions in turn causes reverse-activation of the $2Na^+/Ca^{2+}$ ion exchanger which results in Ca^{2+} overload as shown in Figure 1.3 (Kristian et al. 1998). Increased cytoplasmic Ca^{2+} leads to mitochondrial dysfunction with leakage of pro-apoptotic proteins such as cytochrome C through the opening of mitochondrial permeability transition pores (mPTP) resulting in apoptotic cell death (Kumar et al. 2007). Furthermore, ischaemia causes damage to the electron transport chain (complex I and III) resulting in increased ROS, which also further contributes to mitochondrial injury (Chen et al. 2008; Chen, Hoppel & Lesnefsky 2006). Ischaemia has been shown to suppress transcription and translation, affecting the sarcolemmal dystrophin complex implicated in plasma membrane repair and contraction

apparatus, which further contribute to pathogenesis of ischaemic insult (Rodriguez et al. 2005; Gumerson & Michele 2011).

The restoration of blood flow (known as reperfusion) can mitigate the effects of ischaemia and preserve tissue if performed quickly. Reperfusion therapy was first introduced by Chazov and colleagues in 1975 by the use of streptokinase infusion as a thrombolytic into the coronary arteries of AMI patients. Since then, more potent fibrinolytic and antiplatelet reagents have been developed which reduce MI-related mortalities compared to older generation fibronolytics (Wong & White 2011). These methods have further evolved into more refined approaches, including drug-eluting stents and aspiration thrombectomy with primary percutaneous intervention (Braunwold 2012). Early reperfusion of the ischaemic myocardium results in greater myocardial salvage leading to greater functional recovery and improved prognosis (Ortiz-Pérez et al. 2010).

Reperfusion, although necessary to salvage the ischaemic myocardium from reversible injury, has been recognised to contribute to final infarct size. Jennings and colleagues (1960) observed that 30-60 minutes of ischaemia-reperfusion (I-R) is histologically comparable to that of 24-hour coronary artery occlusion in canine hearts and concluded that reperfusion accelerates development of necrosis (Jennings et al. 1960). Indeed, similar observations have been made in intestinal mucosal lesion formations with 3-hour ischaemia and 1-hour reperfusion resulting in significantly higher injury than 4-hour ischaemia alone (Parks & Granger 1986). For some time, reperfusion injury had been deemed as a laboratory artefact due to accumulation of negative findings in infarct size reduction and in discrepancies in animal models (Kloner 1993; Zahger et al. 1995; see review Ibáñez et al. 2015). However, with the discovery of ischaemic post-conditioning and emergence of pathways that are activated during reperfusion, reperfusion injury is nowadays a widely accepted concept (Zhao et al. 2003; Hausenloy & Yellon 2007).

Similar to ischaemic injury, reperfusion also results in reversible and irreversible injury. Reperfusion can induce ventricular arrhythmia in patients which correlates with significantly lower left ventricular ejection fraction and increased final infarct size when compared to controls (Majidi et al. 2009). Reoxygenation and energization of the ischaemic tissue by reperfusion, compounded by calcium overload from the ischaemic phase, can result in the

development of hypercontracture phenomena, which further exacerbates cellular injury by rupturing adjacent cells (Piper, Garcia-Dorado & Ovize 1998; Ruiz Meana et al. 1999). Similarly, myocardial stunning may also occur as a result of reperfusion, developing due to transient calcium overload. Calcium-dependent proteases may also be activated resulting in degradation of myofibrils leading to contractile depression, although this type of injury is reversible (Bolli & Marban 1999). Cardiomyocyte death is further complicated with the emergence of microvascular dysfunction. Areas of no-reflow prevent arterial blood and pharmacological agents from entering the ischaemic region and the no-reflow phenomena has been estimated to account for approximately 40% of the anterior AMI patients (Ito et al. 1996). Microvascular dysfunction has been shown to correlate with reduced myocardial salvage, increased infarct size, adverse ventricular remodelling and adverse clinical outcomes (Ortiz-Pérez et al. 2010; Ito et al. 1996; Lund et al. 2007). The aetiology of no-reflow involves neutrophil plugs, formation of emboli at capillaries, severe alterations in endothelial cell morphology, oedema and extravasation of blood (Niccolo et al. 2009; Kloner 2011).

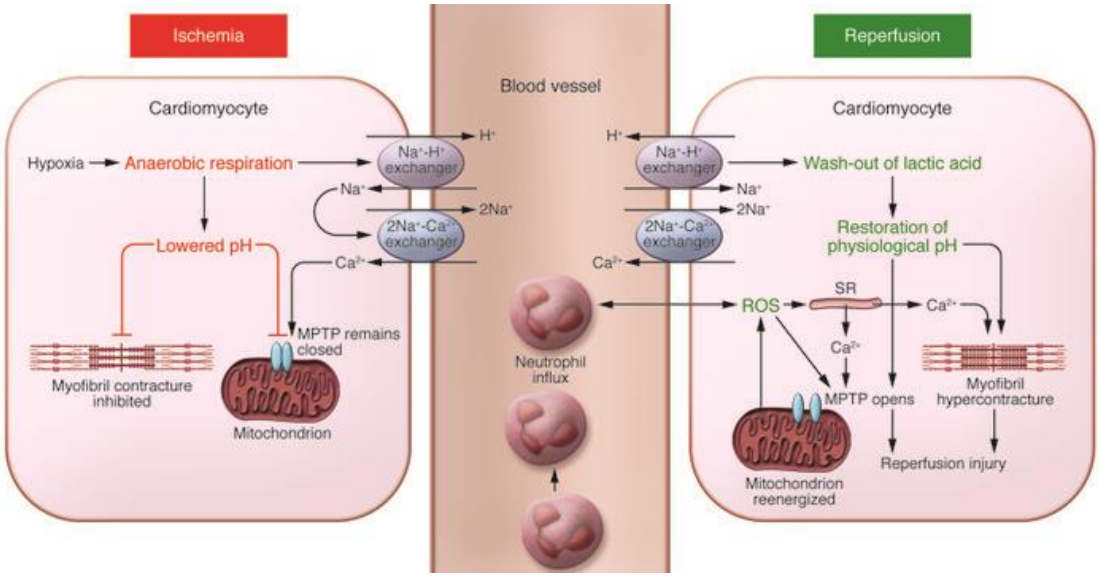


Figure 1.3 Ischaemia induces anaerobic glycolysis resulting in pH decrease and intracellular accumulation of calcium, which results in closure of the mPTP and myofibril contraction inhibition. Upon reperfusion, restoration of pH results in the re-energised mitochondria which resulting in opening of the mPTP. Opening of the mPTP in turn results in production of ROS which damage mitochondrial electron transport chain proteins and serve to attract neutrophils contributing to reperfusion injury (Taken from Hausenloy & Yellon 2013).

1.3 Ischaemia-Reperfusion Models

Three general approaches are available to study I-R injury in animals: 1) *in vivo,* 2) *ex vivo* and 3) *in vitro*. The *in vivo* "open chest" approach first described in 1995 involves tracheotomy incision to establish stable ventilation followed by chest incision to reach the heart and expose the lower anterior descending artery (LAD) (Michael et al. 1995). Prior to chest incision, blood vessels are coagulated using available means followed by series of incisions to expose the pericardium as described in Conci and Metzler (2006). Once the LAD is visualised, a ligature (i.e. PE-10 tubing for I-R) is carefully tied underneath and over the artery with positive occlusion indicated by anterior wall of the left ventricle becoming pale (Tarnavaski et al. 2004). This open chest model permits the study of inflammatory responses as a result of I-R which are lacking in *ex vivo* and *in vitro* methods (Nossulli et al. 2000). Additionally, the chest may be resealed with the heart in place which allows follow-up studies (1-2 week) investigating remodelling of the heart in response to I-R injury (Perez et al. 2009).

The *ex vivo* approach, also known as Langendorff perfusion was popularised by the German physiologist Oscar Langendorff in 1897 for its relative simplicity and the broad spectrum of measurements which can be obtained using this method. This method relies on the cannulation of the aorta for perfusion into the heart with Krebs and Henseleit solution typically used for perfusion (Skrzypiec-Spring et al. 2007). Though seemingly simple, care and a degree of dexterity must be introduced between heart isolation and instrumentation as the heart my inadvertently undergo ischaemic preconditioning or even contusion injuries (Skrzypiec-Spring et al. 2007). A typical Langendorff perfusion follows stabilisation (15-30 min), induction of ischaemia (≈30 min), followed by end of reperfusion (≈45 min) and final infarct measurement by transverse slicing of the heart to expose the left ventricle (Bell, Mocanu & Yellon 2011). The Langendorff method allows the use of many physiological parameters as indicators of recovery, including left ventricular developed and end-diastolic pressure, and measurements of cell death can also be obtained using LDH efflux or triphenyltetrazolium chloride staining (Headrick et al. 2001).

In vitro approaches involve the use primary cardiomyocytes (typically from neonatal or adult hearts) or immortalised cardiac cells in culture. Isolated neonatal cardiomyocytes are easier to isolate and culture compared to adult counterparts, however they are more resistant to

hypoxia/ischaemia and do not express some of the sarcomeric proteins (e.g. α-MHC) typically associated with the adult phenotype (Claycomb et al. 1998). Isolated adult cardiomyocytes strive to resemble closest approach to *in vivo* studies and can be maintained up to two weeks under proper culture conditions. However, these cell cultures need to be continually monitored for signs of deterioration, as *in vitro* adult cardiomyocytes suffer from attrition, lose morphology, reduced t-tubule density and other challenging issues associated with this approach (Louch, Sheehan & Wolska 2011). As an alternative, immortalised cell culture models consisting of H9C2 myoblast cells and HL-1 cardiomyocytes can be employed.

H9C2 cells are neonatal myoblast originally isolated from rat ventricular tissue; they have been shown to resemble similar cardiac hypertrophy response when compared to their primary neonatal counterparts (Watkins, Borthwick & Arthur 2011). H9C2 cells have been suggested as a viable model to study metabolic capacity of the heart as H9C2 cells expressed similar level of cytochrome P450 genes when compared to the rat heart (Zordoky & El-Kadi 2007). However, H9C2 cells do not express gap junctions, caveolae, T tubules or myofibrils with organised sarcomeres, which are associated with adult counterparts. Although they were found to preserve electrical and hormonal signalling pathway found in adult cardiac cells (Hescheler et al. 1991). Alternatively, the HL-1 atrial cardiomyocyte cell line has been suggested to resemble adult primary cardiomyocytes as it expresses many sarcomeric associated proteins (e.g. α-MHC, α-cardiac actin) and lack of expression of any foetal-associated transcripts (Claycomb et al. 1998). Electron micrographic and immunofluorescence studies of high-passage HL-1 cells show these cells remain differentiated after high passages unlike their embryonic counterparts (Claycomb et al. 1998). HL-1 cardiomyocytes have also been used pharmacologically to study ion channels, as they display action potentials and currents similar to that of primary adult cardiomyocytes (Claycomb et al. 1998). In addition, unlike their neonatal counterparts, HL-1 cells are not resistant to I-R injury. HL-1 cells and its parent AT-1 cells have been shown to display ultrastructural characteristics typical of in vivo atrial cardiac muscle cells, including well-organized myofibrils, gap junctions, and atrial-specific cytoplasmic granules (Claycomb et al. 1998; del Carpio et al. 1991). However, there are some divergent instances where HL-1 cardiomyocytes do not resemble adult cardiomyocyte phenotype such as in energy metabolism in HL-1 cardiac cell which show a distinct glycolytic phenotype and possess

mitochondria with deficient respiratory chain complex I (Eimre et al. 2008). Although cardiomyocyte cultures have been shown to be a good model for studying cardioprotective responses, some crucial physiological phenomenon such as hypercontracture and the ensuing inflammation cannot be replicated *in vitro* (Diaz & Wilson 2006). Therefore, their use should be carefully considered and may best serve as a supplementary approach.

1.4 Cardioprotection

As previously discussed, following AMI, timely reperfusion of the ischaemic myocardium can result in preservation of over 50% of the myocardial tissue (Schömig, Ndrepepa & Kastrati 2006). However, reperfusion is a 'double-edged sword' as restoration of blood flow may contribute to as much as 50% of the final infarct size (Jennings et al. 1960). Decades of research have shown that the heart possesses many intrinsic defence mechanisms. These mechanisms can be activated endogenously or exogenously and are capable of reducing or preventing myocardial damage. Cardioprotection can be defined as prevention or reduction of injuries (oncosis, arrhythmias, contractile dysfunction) from myocardial infarction and I-R (Heusch 2013). This phenomenon was first recognised by the use of 'ischaemic-preconditioning' (IPC) technique in canine hearts by Murry and colleagues in 1986. IPC results from the application of brief sub-lethal episodes of I-R before the main lethal ischaemic phase. Following IPC, infarct size was reduced by as much as 75%; the accompanying reperfusion injury was also significantly reduced (Murry et al. 1986). Similarly, several types of 'conditioning' methods have been developed including post-conditioning (IPOST), remote IPC and remote IPOST (Bousselmi, Lebbi & Ferjani 2013). The increased tolerance to I-R injury afforded by these methods is determined in part by the activation of endogenous signalling pathways within the cardiac cells.

Since then, numerous pharmacological manipulations of these same endogenous pathways have also clearly demonstrated to elicit similar cardioprotective responses in both humans and animal models (Hausenloy & Yellon 2009; Sanada, Komuro & Kitakaze 2011). However, in the majority of the cases, this response is triggered by receptor activation at the cell surface which transduce their signals resulting in activation of multiple survival kinases, alterations in gene transcription and changing status of the mitochondrial channels/pores (Vinten-Johansen & Shi 2011). Thus, the current signalling paradigm can be summarised as 1) trigger

phase (e.g. cell surface receptors), 2) mediators (e.g. survival kinases) and 3) end-effectors (e.g. gene expression and mitochondria) as collectively summarised in figure 1.4 (Vinten-Johansen & Shi 2011). It is noteworthy that cardioprotective stimuli can produce two windows of protection, termed first window of protection and second window of protection. The first window of protection relies on immediate changes in proteins, i.e. by phosphorylation, has a greater reduction in infarct size and lasts approximately 2 hours following the application of the IPC. The second window of protection relies on increased transcriptional activity and *de novo* protein synthesis which begins approximately 24 hours and lasts up to 72 hours with a smaller degree of reduction of infarct size (see review Yellon & Downey 2003; Hausenloy & Yellon 2010). Following is a brief summary of cardioprotective signalling, for more details on historical perspective, clinical trials, effects of comorbidities on cardioprotection and pharmacological agents used to induce cardioprotection see Hausenloy & Yellon (2016) and Ferdinandy et al. (2014).

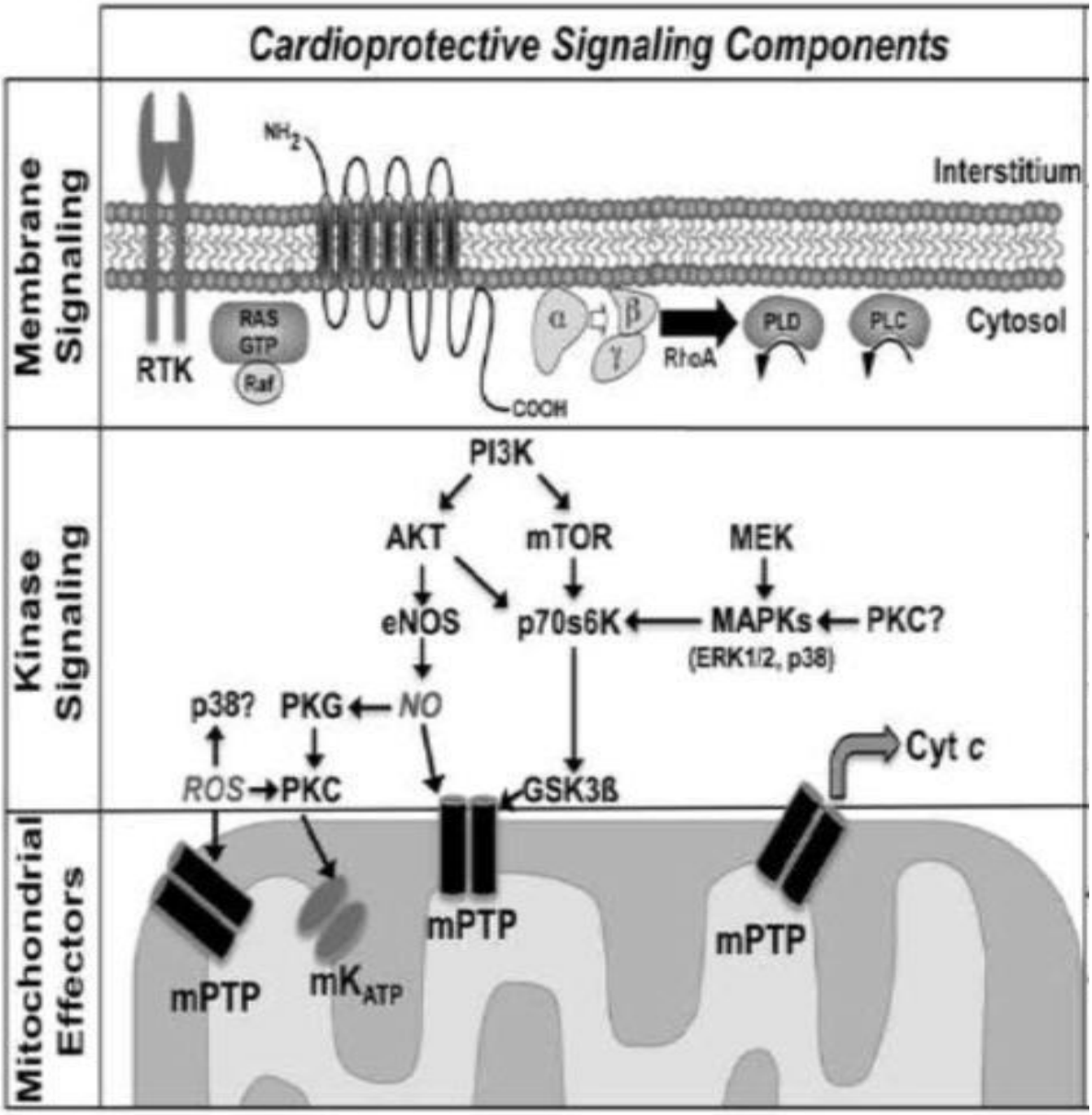


Figure 1.4 Current paradigm of myocardial protective signalling whereby signal transduction from the plasma membrane leads to activation of cystolic kinases (i.e. Akt) affecting mitochondrial targets (mPTP, mK_{ATP}). (Taken from Peart et al. 2014).

1.4.1 The Trigger Phase

The first insight of mimicking IPC pharmacologically was shown by Liu and colleagues in 1991, where they showed that selective A_1 adenosine receptor (A_1AR) agonism reduces infarct size whilst A_1AR antagonism resulted in loss of IPC and A_1AR agonism. Experimentally, several G-protein coupled receptors (GPCRs) such as the adenosine (A_1, A_{2A}, A_{2B}, A_3), opioid (κ and δ subtypes), β-adrenergic (β_1 and β_2 subtypes) and B_2 bradykinin receptors are widely used to promote ischaemic tolerance comparable to that of IPC (Zhan, McIntosh & Lasley 2011; Tanaka, Kersten & Riess 2014; Salie, Moolman & Lochner 2011; Manolis et al. 2010). The GPCR gene family consists of over 1,000 unique members and accounts for approximately 4% of all genes (Insel et al. 2005). The heart expresses approximately 200 different GPCRs and this family represents the largest gene products targeted by therapeutics in clinical use (Devi 2001). In the cardiovascular system, GPCRs control heart rate, vascular tone, contractility, and blood volume. Changes in GPCR expression and signalling feature prominently in many cardiovascular pathologies including AMI, heart failure, hypertension, and cardiomyopathy, thus making them key pharmacological targets (Belmonte & Blaxall 2012). For example, clinically antagonism of β-receptor receptors has been used to treat heart failure since 1975 (Gheorghiade, Colucci & Swedberg 2003). GPCR signalling is complex and involves many proteins. GPCRs are labelled as seven transmembrane receptors that interact with heterotrimeric G-proteins composed of α-, β- and γ-subunits that are GDP bound in the resting state. A brief discussion of GPCR signal transduction will entail as the formation of the second messenger system following receptor activation is crucial for activation of cardioprotective kinases

Agonist binding triggers a conformational change in the receptor, which catalyses the dissociation of GDP from the α-subunit followed by GTP-binding to Gα and the dissociation of Gα from Gβγ subunits. There are several types of Gα subunits these are classified into positive regulators of cAMP ($G\alpha_s$ and $G\alpha_i$ subunits), negative regulators of cAMP ($G\alpha_q$ subunit) and positive regulator of phospholipases C (G_q) (Sanada, Komuro & Kitakaze 2011). A single GPCR can couple to one or more families of Gα proteins such as in the case of A_1AR and the δ-opioid receptor. The importance of the G-protein subunits in relaying cardioprotective signalling is demonstrated by the antagonism of the G_i protein which results in increased susceptibility to I-R injury and become unresponsive to A_1AR agonism (De George et al. 2008; Germack, Griffin

& Dickenson 2004). The formation of the second messengers PIP_3, cAMP and DAG is necessary for activation of the protein kinase family of proteins (PKB/Akt) involved in relaying cardioprotective signalling to mitochondria and the nucleus (Hausenloy & Yellon 2006; Salazar, Chen & Rockman 2007). The DAG and PIP_3 messengers also affect heart rate, contractility and energy metabolism (Xiao et al. 2006). IPC has been shown to enhance DAG expression which is thought to result in activation of PKC, an important mediator of cardioprotection (Cohen et al. 1996). Similarly, PIP_3 generated by PI3K has been shown bring Akt to the membrane and alter Akt its conformation as well as activating PDK which in turn activates variety of effectors such as PKC and p70S6K involved in cardioprotection (Figure 1.4) (see review Murphy & Steenbergen, 2008). It is noteworthy that the specialised microdomain termed caveolae are particularly enriched in PIP_2 and its activator PLC which forms PIP_3 (Lanzafame et al. 2006).

It is now widely believed that the protection afforded by IPC is from autocrine/paracrine release of endogenous ligands for GPCRs such as catecholamine and adenosine released from the ischaemic heart, which becomes focally accumulated upon reperfusion (Liu et al. 1991; Kitakaze & Hori 1998). Indeed, post-conditioning results in reduced endogenous adenosine washout which is attributed to activation of adenosine receptor resulting in reduced infarct sizes compared to controls (Kin et al. 2005). In the clinical setting, pharmacological manipulation of these receptors presents an attractive opportunity in lieu of applying IPC as they can be administered during the infarction and/or reperfusion phase and does not require an invasive procedure (Gross & Gross 2006). Besides reducing infarct size, pharmacological agonism of κ-opioid or the A_1AR also have notable anti-arrhythmic affect, as well as enhancing contractile recovery (Wang et al. 2001; Ashton et al. 2003; Peart & Gross 2004). Stimulation of these receptors results in activation of kinases involved in Reperfusion Injury Salvage Kinase pathway (RISK). To further support this, non-selective adenosine receptor blockers have been shown to reduce IPC and A_1AR agonist mediated phosphorylation of survival kinases including Akt/Protein kinase B and extracellular receptor kinase 1/2 (Erk1/2), linked to increased infarct sizes in these hearts (Solenkova et al. 2006; Germack & Dickenson 2005). In summary, phosphorylation of these kinases are associated with reduced infarct sizes and improved cardiac function whilst antagonism of their upstream components reverses this (Porter et al. 2012).

The importance of receptor activation can be seen with antagonism of these cardioprotective receptors. For example, antagonism of δ_1 opioid receptor results in an infarct size comparable to that of controls (Schultz et al. 1998). It is interesting to note that receptor antagonism within a receptor family, e.g. κ-opioid-receptor antagonism can result in ablation of δ_1 opioid receptor-induced cardioprotection (Peart & Gross 2004). Furthermore, there is evidence of cross-talk between receptors, including the A_1AR and δ-opioid receptor. For example, A_1AR antagonism results in the loss of protection afforded by δ-opioid-receptor agonism and vice versa (Peart & Gross 2003). At the transcriptional level, cross-regulation of receptor mRNA expression can also be seen with A_1AR agonism repressing δ-opioid-receptor transcription and *vice versa* (Pritchard, Peart & Headrick 2008). It has been hypothesised that these receptors dimerise together and is supported by immunoprecipitation studies (Surendra et al. 2013). Similarly, δ-opioid receptor mediated infarct reduction has been shown to be negated by β_2-adrenoceptor antagonism, with co-localisation of these receptors also shown by co-immunoprecipitation (Huang et al. 2007; Jordan et al. 2001).

Besides GPCR to GPCR crosstalk, there is also cross-talk between GPCRs and receptor tyrosine kinases (RTK). RTKs are key regulators of critical cellular processes such as cell proliferation and differentiation, survival and metabolism, migration and cell cycle control (Lemmon & Schlessinger 2010). RTKs have been characterised with 58 known RTKs identified in humans to date (Lemmon & Schlessinger 2010). Similar to GPCRs, RTKs are also present in the plasma membrane and numerous endogenous ligands for RTKs have been implicated in cardioprotective signalling. Namely, these include fibroblast growth factors, vascular endothelial growth factors and extracellular growth factors (Hausenloy & Yellon 2009; Roth & Patel 2011). RTKs are generally activated by ligand-induced dimerization, which brings its two subunits in close proximity to each other. The activated RTKs subsequently recruit second messenger systems through Grb2 and Shp2, which activate RAS and Erk1/2 or DAG and IP3 in a similar manner to GPCR activation (Lemmon & Schlessinger 2011). Consequently, depending on the second messenger system, activation of RTKs results in broad category of responses including apoptosis, inflammation, hypertrophic response and fibrosis (Detillieux et al. 2003). Activation of epidermal growth factor receptor (EGFR) has recently been shown to be involved in cardioprotection, with evidence that EGFR transactivation triggers β_1-adrenergic, A1AR, δ-opioid-receptor and acetylcholine mediated cardioprotection (Noma et al. 2007;

Pritchard et al. 2011; Fryer et al. 2001; Krieg et al. 2002). A_1AR agonism via CCPA has been shown to increase phosphorylation of EGFR, Erk1/2 and Akt, which are blocked by EGFR antagonism (Pritchard et al. 2011). GPCR-EGFR transactivation is mediated by at least two mechanisms, either through matrix metalloproteinases (MMPs) or the β-arrestin family of proteins (Pritchard et al. 2011; Noma et al. 2007).

MMPs are large family of proteins that are able to digest extracellular matrix proteins such as collagen and fibronectin (Visse & Nagase 2003). MMPs have been shown to cleave the EGFR ligand Hb-EGF, which in turn has been shown to activate EGFR and induce cardioprotection (Cheng, Xie & Raufman 2007). Pharmacological inhibition of MMP results in the loss of adenosine-mediated cardioprotection and an associated decrease in Ek1/2 and Akt phosphorylation (Pritchard et al. 2011). Another important regulator of GPCR and EGFR transactivation has been shown to involve β-arrestins of which there are two known subtypes, β-arrestin 1 and β-arrestin 2. The β-arrestin family of proteins has been implicated in the uncoupling G-proteins from their receptors, function as scaffold proteins that interact with several cytoplasmic proteins, and link GPCRs to intracellular signalling pathways including MAPK cascades (Ma & Pei 2007). β-arrestin 1/2 co-precipitate with over 300 distinct proteins, implicating that β-arrestins might link GPCRs to a wide range of signalling pathways (Xiao et al. 2007). β-arrestins have also been implicated in desensitisation, sequestration, intracellular trafficking of GPCRs and interacts with transcription factor such as NF-κB (Luttrell & Lefkowitz 2002; Ma & Pei 2007). Desensitisation of GPCR is initiated by the phosphorylation of the receptor through GRKs and is dependent on the presence of β-arrestins. In the case of the β2 adrenergic receptor, β-arrestins have been shown to enhance the binding affinity GRK to GPCRs (Lohse et al. 1993). Intracellular trafficking of GPCRs through endocytosis has also been shown to involve the recruitment of β-arrestins (Reiter & Lefkowitz 2006).

1.4.2 The Mediator Phase

Activation of cardioprotective GPCRs results in changes in phosphorylation status of downstream signalling kinases including the members of MAPKs, Erk1/2 and p38 MAPK, serine/threonine kinases such as various isoforms of PKC, Akt and JAK/STAT which have pro-survival effects on the cell. Expression and phosphorylation status of MAPKs, Erk1/2, p38 MAPK and the Akt serine/threonine kinase are typically used as indicators of cardioprotection (via immunoblotting) as well. A_1AR, δ-opioid-receptor and bradykinin B_2 activation have all been shown to elicit cardioprotection through phosphorylation of Erk1/2, Akt and p38 MAPK (Peart & Gross 2005; Pritchard et al. 2011; Dana et al. 2000; Peart et al. 2007). Conversely, pharmacological antagonism of these receptors results in reduced phosphorylation of these kinases and their distal targets. Generally, the phosphorylation status of these kinases does not change significantly during ischaemia, however significant activation is observed within 5 minutes of reperfusion (Fryer et al. 2001a; Fryer et al. 2001b). Hence, the classification convention for these kinases has been suggested to be Reperfusion Injury Kinase Pathway or RISK (Hausenloy, Tsang & Yellon DM 2005). Pharmacological antagonism of these kinases (p38 and Erk1/2) ablates IPC, GPCR and RTK mediated phosphorylation of these prosurvival kinases resulting in increased infarct sizes (Hausenloy et al. 2005; Fryer, Hsu & Gross 2001; Chai et al. 2008). Phosphorylation of Erk1/2 results in the translocation of Erk1/2 to the nucleus where it activates many stress-responsive transcription factors including c-Fos and c-Myc (Plotnikov et al. 2011). Activation of Akt leads to inactivation of the pro-apoptotic proteins (e.g. GSK3β and BAD) and influences transcription of several translation factors and ribosomal proteins (Fang et al. 2000; Sussman et al. 2011; Luna-Ortiz et al. 2011). PKC with its various isoforms is involved in various modes of cardioprotection including IPC, pharmacological and other forms of conditioning (Simkhovich, Przyklenk & Kloner 2013). Depending on the isoforms, PKCs can be cardioprotective or cardiodetrimental, as observed with PKCε and PKCδ (Sivaraman et al. 2009). PKC isoforms targets within the cell include eNOS, the mitochondrial K_{ATP} channels and permeability transition pores and L-type Ca^{2+} channels (Ohnuma et al. 2002; McHugh et al. 2000 Budas; see review Churchill & Mochly-Rosen 2007).

1.4.3 The Effector Phase

Regardless of the original stimulus, all cardioprotective signalling converge on the mitochondria and the nucleus leading to reduction in cell death. The heart is a high-energy organ due to its reliance on aerobic mitochondrial metabolism; it is also consequently vulnerable to a decrease in oxygen supply as encountered during ischaemia. Besides being the energy powerhouses in the heart, mitochondria are also pivotal decision-makers regarding cell survival. More specifically, mitochondria are capable of release of pro-apoptotic factors such as cytochrome C following I-R injury (Borutaite et al. 2003). Both ischaemic stress and ROS results in damage and inhibition of mitochondrial proteins such as ATPase, as well as resulting in increased entry of Ca^{2+} into the mitochondria which causes mitochondrial swelling (Chen et al. 2008; Lee et al. 2012). Preservation of mitochondria following I-R is a key feature of several cardioprotective methods and is associated with enhanced recovery (Sun et al. 2015; Chen et al. 2007). Pharmacological manipulations have shown notable cardioprotective targets in the mitochondria including the mitoK$_{ATP}$ channel, voltage-dependent anion channels (VDAC), mitochondrial permeability transition pore (mPTP) and glycogen synthase kinase-3β (GSK3β).

Cardioprotective stimuli such as IPC have been shown to open the mitoK$_{ATP}$ channel which is thought to have several consequences including membrane depolarisation, matrix swelling, enhanced respiration and reduced Ca^{2+} overload (Gross & Fryer 1999). Whereas, agonism of the mitoK$_{ATP}$ channel have been shown to reduce infarct size (Pain et al. 2000). Recent evidence suggests that the mitoK$_{ATP}$ channel is not an end-effector, with the channel playing a more upstream role. Opening of the mitoK$_{ATP}$ channel has been suggested to orchestrate a series of events including ROS formation in the matrix, eventually leading to the closure of the mPTP (Cohen & Downey 2008; Garlid et al. 2009). PKCε appears to be the only isoforms that opens the mitoK$_{ATP}$ as pharmacological antagonism of it does not block PKCε-dependent mitoK$_{ATP}$ opening (Costa et al. 2005).

The mPTP is a non-specific channel thought to span both mitochondrial membranes and is responsible for maintaining the H^+ gradient for ATP synthesis. The mPTP remains closed during ischaemia, however the accumulation of Ca^{2+}, ROS and inorganic phosphate during reperfusion results in the activation of the mPTP (Baines 2009). Inhibition of the opening of

this pore by administration of cyclosporin A and IPC in I-R hearts results in mPTP closure, leading a reduction in mitochondrial Ca^{2+} loading, stunning and necrotic injury (Griffiths & Halestrap 1993; Javadov et al. 2003). Both Akt and Erk1/2 can also inhibit the opening of the mPTP following I-R by activation of downstream mediators such as ornithine decarboxylase/polyamine system following I-R (Zhang et al. 2014). GSK-3β has been implicated as an important mediator of mPTP-mediated cardioprotection as phosphorylation of GSK3β prevents mPTP opening (Juhaszova et al. 2004). The exact mechanism of GSK3β-mediated prevention of mPTP opening is still unknown, although several possibilities have been suggested (see review Miura & Tanno 2010). The impact of VDAC in I-R resistance is also currently unknown, as its closure on apoptosis status has been met with contrasting results (see review Das & Steenbergen 2012). Studying VDAC has been made difficult due to lack of specific pharmacological tools and embryonic lethality observed with VDAC2-deficient mice (Cheng et al. 2003). However, knockdown of *Vdac2* using RNA interference in mouse embryonic fibroblasts resulted in increased cell death following oxidative stress, whilst *Vdac1*- and *Vdac3*-knockdown showed no change in susceptibility (Baines et al. 2007). Similarly, *Vdac2* is necessary for mitochondrial translocation of GSK3β as knockdown of *Vdac2* (but not *Vdac1* and *Vdac3*) results in mPTP opening under oxidative stress (Tanno et al. 2014).

Besides affecting mitochondria, alterations in nuclear signalling is pivotal for cardioprotection as studies have shown that Erk1/2, Akt, MAPK isoforms and even G-protein subunits (e.g. G_α) interact with transcription factors and chromatin regulatory molecules such as histone deacetylases (Vaniotis, Allen & Hebert 2011). Nuclear signalling is of particular importance for late preconditioning (the second window of protection) which alters the expression of transcripts encoding for cardioprotective proteins, including iNOS and COX-2 (Hausenloy & Yellon 2010). Activation of p38 MAPK has been shown to result in increased NFκB translocation to the nucleus and is an important mediator of A_3AR agonism and IPC (Das et al. 1999; Zhao & Kukreja 2002; Xuan et al. 1999). NFκB has been shown to impact the transcription of diverse groups of genes involved in apoptosis, cell development and angiogenesis (Tranter et al. 2010). The activation of Janus kinase (JAK) and signal transducer and activator of transcription 3 (STAT-3) from the Survivor Activating Factor Enhancement (SAFE) pathway have also been implicated in nuclear signalling following pharmacological preconditioning (Lecour et al. 2005). Activation of the SAFE pathway also leads to JAK-STAT-

mediated transcription of genes encoding Bcl-2, Bcl-xL, Mcl-1, Fas, cyclin D1, cyclin E1 and p21, which are involved in cell survival and proliferation (Lecour & James 2011). More recently, a sub-population of phospho-Erk1/2 has been shown to be directed to the mitochondrial membrane, leading to increased phosphorylation levels of mitochondrial proteins and inhibition of mPTP opening (Hernández-Reséndiz & Zazueta 2014). In summary, cardioprotective signalling converges on the mitochondria targets such as the mitochondrial K_{ATP} channels, and mPTP leading to preservation of mitochondria which is associated with improved recovery (Fridolfsson et al. 2012). Nuclear expression profile is also affected which causes increased *de novo* synthesis of cardioprotective antioxidant enzymes, COX2 and heat shock proteins (Yellon & Downey 2003).

1.5 Loss of Cardioprotection in the Aged Heart

The general process of ageing is associated with cardiac susceptibility to disease and responsiveness to therapy. The aged heart is most likely to suffer I-R insult, with approximately 75% of infarcts occurring in those 65 years of age or older (Mariani et al. 2000). In the ageing mouse heart, 75% of ischaemic intolerance develops before 12-months ('middle-aged') and precedes morphological changes (Willems et al. 2005). This reduction in ischaemic tolerance has been shown to be independent of ischaemic duration, and collateral flow, further indicating an ageing-related decline of intrinsic myocardial resistance to ischaemic injury (Willems et al. 2005; Fenton et al. 2000). Furthermore, both ischaemic and pharmacological preconditioning of animal and human hearts have yielded mostly negative results, which fail in the aged hearts unlike in the young counterparts (Boengler, Schulz & Heusch 2009; Sivaraman & Yellon 2014). Similarly, protection due to preinfarction angina, thought to mimic IPC resulting in a lower incidence of in-hospital mortality, heart failure, cardiogenic shock, and myocardial necrosis observed in younger patients, has been reported to be both retained and lost in the elderly (Ishihara et al. 2000; Loubani, Ghosh & Galiñanes 2003). As the therapeutic promise of cardioprotective methods has not been realised due to poor clinical translation, it has been suggested that there is a bias to publication of positive rather than negative findings in the scientific literature, which in turn may inadvertently bias views on the efficacy of a particular response (Peart & Headrick 2009). For example, the AMIDSTAD II trial showed that adenosine treated group anterior MI group resulted in 67% reduction (vs. placebo) in infarct size although it did not reduce the primary end point of survival (Ross et al. 2005). The cellular mechanisms and molecular basis of these altered responses in the aged hearts are only just beginning to be understood. Furthermore, confounding effects of age, sex and comorbidities are also beginning to be understood, further unravelling the stress-intolerant aged phenotype and will be briefly discussed in the following sections.

1.6 Ageing in the Heart

1.6.1 General Characteristics

Ageing affects cardiomyocytes at several subcellular and molecular levels including telomere shortening, mitochondrial dysfunction, changes in transcription and translation (Kovacic et al. 2011; Ashton et al. 2006). There are also notable physiological changes, linked to molecular changes including cardiac hypertrophy and left ventricular fibrosis (Sangaralingham et al. 2011). For example, aged-related loss of ventricular mass is compensated by cardiomyocyte hyperplasia and fibrosis due to continual loss of cardiomyocytes (Anversa et al. 1990). It is noteworthy that these histological evident changes occur with cellular senescence (>19-21 months old) but not in the middle-aged (10-12 months old) hearts (Anversa et al. 1986). Ageing is also accompanied by notable vascular changes such as arterial wall thickening, endothelial dysfunction, and increased systolic blood pressure accompanied by decreased diastolic blood pressure which increases the workload of the heart (See review; Kovavic et al. 2011b). Cardiomyocyte ageing has been shown to lead to alterations in telomeres, telomerase activity, oxidative stress response, and expression of $p16^{INK4a}$ and p53 which are determinants of cellular senescence, impairing cell growth and promoting cell death (Torella et al. 2004). Replicative senescence of the cardiomyocyte is paralleled in the cardiac stem cell population, which also becomes mostly senescent (c-kit^{POS}) although some telomerase-competent cardiac stem cells are still able to differentiate into cardiomyocytes (Chimenti et al. 2003; Gonzalaz et al. 2008). However, continual replacement of the dying cardiomyocyte population with a declining pool of available cardiac stem cells leads to a progressive depletion of the growth reserve of the heart (Chimenti et al. 2003). There are notable sex-related differences in the accumulation of senescent cardiomyocytes, 0.68% per year in women and 0.89% per year in men (Kajstura et al. 2010). Furthermore, the female heart has been shown to possess a larger pool of functionally competent cardiac stem cells and younger myocytes than the male myocardium (Kajstura et al. 2010).

The heart is enriched with mitochondria, which provide 70% of the ATP through oxidative phosphorylation (Saraste 1999). Mitochondrial dysfunction is central to the theory of ageing and was noted for its role in ROS generation linked to the main cause for age-related damage in the early 1970s (Harman 1972). Indeed, the decline of cardiac performance correlates well

with decline of cardiac mitochondria as shown in Table 1 of Dai, Rabinovitch & Ungvari (2012). Age-related mitochondrial changes have been shown to include increased ROS, impaired oxidative phosphorylation, reduced ATP generation, and impaired fatty acid oxidation. These dysfunctions are thought to arise from increased instability of the mitochondria by increased mitochondrial DNA mutations and reduced autophagy (Brink et al. 2009; Marín-García, Pi & Goldenthal 2006). The effects of increased mitochondrial DNA mutations can be mimicked by mice models employing homozygous mutation of mitochondrial polymerase gamma (DNA proofreading enzyme) with these mice showing several age-related diseases such as greying and loss of hair, osteoporosis, sarcopenia and shortened life span of approximately 15 months (Trifunovic et al. 2004). Another prominent change that has been shown to contribute to ageing is the reduced efficacy of mitochondrial autophagy, a self-recycling process where faulty mitochondria are removed. To highlight the role of autophagy in maintenance of cardiac structure and aging, cardiac-specific Atg5 (autophagy-related gene 5)-deficient mice show disorganised sarcomere structure and begin to die after the age of 6 months (Taneike et al. 2010).

Ageing has been shown to result in significant alterations in transcriptional response in both normoxic and post-ischaemic hearts which impact crucial cardioprotective signalling networks including GPCR, WNT, JAK-STAT, and MAPK (Ashton et al. 2006). Although cardiomyocytes occupy 75% of the ventricular tissue volume, they have been shown to account for only 30–40% of the total number of cells in the adult heart with fibroblast population being second highest non-muscle type cell (Nag 1980). The ageing heart displays dysfunctional mesenchymal fibroblasts, which result in upregulated collagen deposition from 7% in the young heart (4-week old) to 20% in the senescent heart (88-week old) (Cieslik et al. 2013; Eghbali et al. 1989; see review Biernacka & Frangogiannis, 2011). All of these have been estimated to result in loss of 35% of cardiomyocytes and a 20% decrease of sinoatrial nodal pacemaker cells due to apoptosis, necrosis and potentially autophagy (Olivetti et al. 1991; Chien & Karsenty 2005). It is noteworthy to mention that cardiac ageing in the murine models mentioned previously resembles the age-related cardiac changes in healthy human population (Dai & Rabinovitch 2009). For a summary of cardiovascular ageing see Lakatta et al. (2015).

1.6.2 Clinical Trials

Since the discovery of IPC, more than 3,000 cardiovascular investigations have been published to understand the underlying mechanisms of IPC and clinical translation of IPC (Peart & Headrick 2009). Furthermore, the discovery of post-conditioning revitalised the pursuit of cardioprotective methods in the clinical setting as it can be applied during reperfusion (Zhao et al. 2003). As mentioned previously, pharmacological recruitment of cardioprotective receptors can also yield in reduction in I-R injury as shown by various animal models and pharmacological agonists (Hausenloy & Yellon 2009; Sanada, Komuro & Kitakaze 2011). In concordance with the ageing animal models, the results with pharmacological activation of cardioprotection signalling are mostly disappointing as reduced infarct size was seen in some clinical studies provided no prognostic benefits (Boengler, Schulz & Heusch 2009; Heusch 2013). For example, A_1AR agonism in 2 clinical trials, Acute Myocardial Infarction Study of Adenosine (AMISTAD-I and AMISTAD-II), consistently showed reduced infarct size (33% and 67%, respectively) without improved survival (Mahaffey et al. 1999; Ross et al. 2005). However, issues have been raised including whether the bolus was sufficient enough to elicit a response during the reperfusion phase, which was also limited by the hypotensive side effect of adenosine, and its half-life (Kloner et al. 2013). It was later found in *a post-hoc* analysis that 3 hours of adenosine administration as an adjunct to reperfusion therapy within the first 3.17 h onset of AMI (anterior ST-segment elevation) enhances early and late survival, as well as reducing the clinical endpoint of death or congestive heart failure at 6 months (Kloner et al. 2006). This is in agreement with animal experiments that even preconditioning methods cannot elicit a cardioprotective response after 3 hours of ischaemia (Murry et al. 1991).

Similar to pharmacological trials, post-conditioning trials have also produced mostly negative results, although a single trial has shown a reduction in infarct size or a trend toward a reduction (see review Kloner 2013). Phase III clinical trials (CIRCUS) inhibiting opening of mitochondrial permeability transition pore with Cyclosporin-A is also underway with small pilot studies in the context of AMI and CABG showing promising results (Hausenloy et al. 2014; Piot et al. 2008). Despite these failures, there is still optimism of cardioprotective methods as an adjunctive therapy for reducing reperfusion injury although the great promise of cardioprotection lies in salvaging the ischaemic myocardium during the infarction process

when the availability of salvageable tissue is large (Heide & Steenbergen 2013). Indeed, the translation of experimental and pre-clinical data into clinical data to this data has not been fully realised despite the large body of work. More recently, a multicentre randomized controlled trial called CAESAR has been developed to evaluate potentially cardioprotective therapies thereby sparing millions of dollars and years of work involved in performing clinical trials of agents (Jones et al. 2015). More specifically, the use of CAESAR protocols has been shown to produce reproducible results in mice, rabbit and pig models in assessment of potential infarct-sparing therapies (Jones et al. 2015). However, as mentioned by the its authors, another complication encountered with clinical trials is the existence of multiple comorbidities and the lack of aged models which have both been shown to affect cardioprotective signalling and are not replicated in animal models. For a summary of the clinical trials of cardioprotection see Heusch (2013).

1.6.3 Effect of Comorbidities and Medications

Ageing hearts with IHD are also associated with multiple underlying diseases, thus necessitating efficacy of preconditioning stimulus or other protective modalities be effective across age group sand disease. Unfortunately, in animal models multiple comorbidities and current drug use does not accurately reflect the clinical scenario of AMI which may help partially explain the lack of clinical progress. Indeed, increasing number of comorbidities has been associated with higher 30-day and 1-year post admission death rates in patients hospitalized with AMI (McManus et al. 2013). Thus, another possible underlying condition limiting the efficacy of cardioprotection in the aged hearts is the existence of multiple comorbidities. These comorbidities have been shown to include atrial fibrillation, heart failure, prior MI, diabetes mellitus, hypertension and stroke (McManus et al. 2013).

Due to its growing near epidemic prevalence in Australia and the rest of the world, Diabetes mellitus will be discussed briefly here as an example (for further details see review McCafferty et al. 2014). In 2013 in Australia, there were approximately 50% of deaths due to Type 2 Diabetes with IHD as an associated cause of death (ABS 2013). Despite its growing prevalence, there is still paucity of data investigating both myocardial conditioning in ageing and diabetic cohorts (Przyklenk et al. 2011). Diabetes is associated with larger infarct sizes and poor

outcomes when compared with non-diabetic patients (Haffner et al. 1998; Stevens et al. 2004). Consistent with experimental findings, Diabetes mellitus has been shown to block the protective effects of IPC, angina, anaesthetic and other forms of pharmacological conditioning (Ishihara et al. 2001; Tai et al. 2012; see review Peart & Headrick 2009). Mechanisms of this reduced protection in diabetic hearts have shown to involve reduced phosphorylation of survival kinases such as JAK/STAT, PI3k, MAPK and GSK3β by various forms on conditioning methods (Gross, Hsu, & Gross 2007; Tai et al. 2012; Whittington et al. 2013). Therefore, the imposition of the diabetic heart on top of the already failing ageing heart may further exacerbate age-related dysfunctional signalling in the heart. Diabetic animal models are also a major hurdle as it has been suggested that none of the models accurately reflect the human pathology of diabetes and have yielded conflicting results (see review Whittington et al. 2012). Use of medications such as β-blockers, statins, nitrates, and antidiabetic drugs for the comorbidities have also have been shown to impact cardioprotective signalling in certain cases (Ferdinandy et al. 2014). Sulfonylurea, an antidiabetic drug, has been associated with an increased risk of in-hospital mortality among diabetic patients undergoing coronary angioplasty for acute myocardial infarction whilst it has no negative effects on the long-term survival (Garratt et al. 1999; Meier et al. 2003).

1.6.4 Sex-Related Differences

As previously mentioned, males are twice as likely to have a heart attack when compared to females (AIHW 2015). Historically, upon the clinical presentation of IHD, women have been treated less aggressively than men, as woman were often misdiagnosed or significantly less likely to report chest pain or discomfort compared with men (Tobin et al. 1987; Canto et al. 2007). Nowadays, although women are less likely to be admitted to coronary care units, the overall treatment is as intensive for women as it is for men (Alfredsson et al. 2009). In certain instances, there are notable differences in pharmacotherapy for woman with IHD. For example, women are less likely to receive aspirin or glycoprotein IIb/IIIa inhibitors and are less often discharged on aspirin or statin (Akhter et al. 2009; see review Ostadal & Ostadal 2012). Due to their older age at clinical presentation of IHD, women are more likely to have a history of hypertension and diabetes, which as previously discussed worsens the outcomes of

IHD (Alfredsson et al. 2007). Although it was generally believed that males displayed greater IHD-related mortality, multivariate analysis correcting for age and co-morbidities show these apparent sex-related mortalities to disappear (Dey et al. 2009). However, there are notable sex-related responses to AMI/I-R both clinically and experimentally. Compared to males, the female myocardium appears resistant to variety of stressors such as ischaemic damage and associated arrhythmias, stress-induced hypertrophy and heart failure (Bell et al. 2013). Consistent with experimental findings, females display reduced prevalence of CVD/IHD, developing IHD on average 10 years later than males, with myocardial infarction occurring 20 years later (Willems et al. 2005; Duvall 2003; Bassuk & Manson 2010). There are also notable differences in myocardial salvage using PCI following AMI between males and females. More specifically, being female has been shown to be an independent predictor of greater myocardial salvage (0.64 vs. 0.50) after PCI (Mehilli et al. 2005). Despite greater myocardial salvage, four-year mortality studies suggest that following multivariate analysis, there are no sex-related differences in mortality and cardiac morbidity after PCI (Tillmanns et al. 2005; see review Bucholz et al. 2014). As discussed later in Chapter 4, the mechanism of this protection in the female heart is thought to include a dependence on estrogen receptor signalling.

1.6.5 Molecular Dysfunction in the Aged Heart

As previously discussed, the ageing heart is more susceptible to damage from I-R relative to the younger heart. The aged heart displays diminished functional capacity due to fibrosis, abnormal calcium handling as well an increased susceptibility to damage (Biernacka & Frangogiannis 2011; Zhou, Lakatta & Xiao 1998). In mice, aged hearts also demonstrate impaired tolerance to I-R as early as 8 months (mature) without any morphological changes (Willems et al. 2005). In addition, aged hearts also do not respond to pharmacological and ischaemic preconditioning (Headrick et al. 2003). The exact mechanism of attenuated cardioprotection in the aged heart is currently unknown. It has been hypothesised that the GPCR system is only partially inactivated by ageing whilst the RTK-mediated system is severely impaired (Yeo & Park 2002). This lack of protection in the aged heart is not likely due to sole receptor dysfunction; for example, A_1AR expression has been shown to be similar in young and aged hearts (Headrick et al. 2003). However, β_1-adrenergic receptors have been shown to be decreased in aged hearts (Cerbai et al. 1995). G-protein coupling mechanisms are also

affected with ageing as shown for the A_1AR and β_1-adrenergic receptor. The basal coupling of Gα with A_1AR has been shown to decrease by approximately 20% (Cai et al. 1997). Whilst A_1AR agonism in the aged hearts resulted in 50% less coupling when compared to young counterparts (Cai et al. 1997). A similar decrease in β-adrenergic responsiveness with ageing involving decreased agonist binding of β_1-receptors, uncoupling of β_2-receptors, and altered G protein-mediated signal transduction is observed in human hearts (White et al. 1994; see review Ferrara et al. 2014). RTKs also show aged-related impairment, as EGFR receptor expression as well as its autophosphorylation is significantly reduced (80%) in senescent fibroblasts, although the status of RTK signalling is unknown in the heart (Tran et al. 2003).

At the mediator level, crucial proteins and kinases that trigger cardioprotection such as Akt Erk1/2 and p70S6k are present and functional in the aged heart, similar to levels seen in the young counterparts. Rather, there is an age-related loss of activation of p38 MAPK (Peart et al. 2007). When p38 MAPK is directly activated (anisomycin), phosphorylation of p38 as well as its downstream target HSP27 is restored accompanied by enhanced recovery in these hearts (Peart et al. 2007). Age-associated impairment of p38 MAPK signalling has been observed in the aged rat brain as well as in aged hypertensive rats and human end stage heart failure (Lemke et al. 2001; Aoyagi & Izumo 2001; Zhen et al. 1999). Pharmacological PKC (α, δ and ε) activation is impaired as PKC agonism, reduces infarct size in young but not aged hearts (Schulman, Latchman & Yellon 2001). This may be due to impaired translocation of PKC isoforms, which display differential expression and localisation with ageing (Tani et al. 2001; Hunter & Korzick 2005). The failure of translocation of the PKC isoforms may be due to age-related decrease in their scaffolding protein, receptors for activated C-kinase (RACKs), which are also decreased in the aged brain leading to impaired translocation of PKC isoforms (Korzich et al. 2001; Pascale et al. 1996). The failed activation of these kinases (Erk1/2, PKC, etc.) in the aged heart may result in impaired phosphorylation of GSK3β, which may affect the distal targets of GSK3β including the mPTP.

Similar to GPCR and PKC insensitivity, pharmacological preconditioning targeting mPTP and mitoK_{ATP} channel also fail in the aged hearts. More specifically, aged hearts display insensitivity to cyclosporine A, which inhibits opening of mPTP in the young heart but not in the aged heart, leading to a significant increase in infarct size (Liu et al. 2011). Similarly, aged hearts are also insensitive to pharmacological activation (via diazoxide) of mitochondrial K_{ATP} and display reduced recovery and enhanced oncosis compared to young counterparts (Peart

et al. 2014). Similar to unperturbed levels of survival kinases, the expression of GSK-3β is similar between the young and aged hearts, however aged hearts display >2-fold increase in phospho-GSK-3β (Kinnard et al. 2005). Aged hearts are also insensitive to direct GSK-3β inhibition (SB-216763), which reduces I-R injury in the young but not aged hearts (Zhu et al. 2011). The mechanism behind the aged-related insensitivity to GSK-3β pharmacological inhibition is unknown, although several hypotheses have been suggested (See *Zhu* et al. 2011). Regarding the enhanced phosphorylation status of GSK-3β in the aged heart, as phospho-p70s6K inhibits GSK3β, it is conceivable that declining expression of phospho-p70s6K may be responsible for elevated phospho-GSK3β in aged tissue (Sutherland, Leighton & Cohen 1993; Peart et al. 2014).

As a proof of concept, cardioprotection in the aged heart can be restored as shown with transgenic approaches such as A_1AR overexpression, pharmacological approaches such as sustained ligand preconditioning (SLP) and caloric restriction (Headrick et al. 2003; Kidd et al. 2010; Peart & Gross 2004; Shinmura, Tamaki & Bolli 2008). These methods have been shown to display a cardioprotective phenotype as indicated by reduced oncosis and improve post-ischaemic recovery, the ideal outcomes of cardioprotective interventions. A_1AR overexpression reduces necrosis, contractile dysfunction and the incidence of tachyarrhythmia with and without the addition of adenosine in aged hearts (Headrick et al. 2003). It is noteworthy that A_1AR overexpression produces a more cardioprotective phenotype in the young heart whilst aged hearts display enhanced cardioprotection compared to their wild-type aged counterparts (Headrick et al. 2003). Thus far, the most characterised mechanism of the aforementioned interventions is SLP. Interestingly, SLP does not involve canonical protective RISK signalling elements, but induces non-canonical transcriptional changes involved in mediators of inflammation/immunity, sarcomere function, and cardiovascular growth and development (Ashton et al. 2013). The mechanisms of SLP protection appear different to conventional δ-opioid receptor agonism, as depletion of specialised microdomains in the plasma membrane does not affect the efficacy of SLP (See Hoe et al. 2014). Although caloric restriction does not improve ischaemic tolerance in the senescent hearts, it does attenuate the age-associated changes in the heart and major vessels such as collagen deposition in the heart and aorta (Ahmet et al. 2011). Microarray analysis of caloric restricted hearts show gene expression changes related to preserved fatty acid metabolism, reduced endogenous DNA damage, decreased innate immune activity, apoptosis

modulation, and a marked cytoskeletal reorganization (Lee et al. 2002). Taken together, these studies suggest that while the machinery to affect cardioprotection is present, there is an age-related impairment in the organisation and regulation of the stress activated signalling cascade. Recent evidence involving the organisation and sequestering of signalling molecules in specialised microdomains termed caveolae have shown additional evidence of this (Kidd et al. 2010; Peart et al. 2014).

1.7 Lipid Rafts and Caveolae

The plasma membrane of a cell is not a homogenous sea of phospholipids, but consists of highly organised microdomains known as lipid rafts. Lipid rafts display a unique biochemistry compared to the rest of the plasma membrane as they are particularly enriched in gangliosides, sphingomyelin and cholesterol rendering them detergent-resistant. Due to enrichment of cholesterol, lipid rafts exhibit less fluidity compared to the surrounding plasma membrane (Pike 2003). The sizes of lipid rafts can vary, but are generally considered to be 50nm in diameter, of which there can be 10^6 rafts in a plasma membrane with each raft carrying about 20 protein molecules (Simons & Ehehalt 2003). Lipid rafts can also be enriched in particular group of proteins such as flotillin, which have variety of roles depending on the cell type (Figure 1.5) (Otto & Nichols 2013). Currently there is no universal consensus on how many types of lipid rafts there are, although a model consisting of: 1) rafts, 2) clustered rafts, 3) detergent resistant membranes and 4) caveolae has been proposed (Simons & Toomre 2000). Caveolae were initially identified by Palade and colleagues in the early 1950s, although this was not published until much later when caveolae were described as 50-100 nm "*flask shape and elongated neck-like invaginations*" in blood capillary of rat tongue (Palade & Bruns 1968). Caveolae can occupy as much as 50% of the plasma membrane in some cell types, with adipocytes estimated to have as many as a million caveolae per single cell (Thorn et al. 2003). Unlike lipid rafts, which are continuous and morphologically indistinguishable from each other, caveolae are invaginations within the membrane and are therefore distinguishable morphologically to the other three types of lipid rafts described above (see Figure 1.5).

Caveolae have been shown to participate in a variety of cellular processes including vesicle trafficking, cholesterol homeostasis, tumour suppression and signal transduction. The selective entry of some bacterial toxins and viruses including SV40 into cells has shown to involve caveolae instead of clathrin (Norkin 2001; Pelkmans, Puntener & Helenius 2002). Similarly, some proteins have been shown to endocytose through caveolae (Razani et al. 2001). Clathrin-mediated endocytosis and caveolae-mediated endocytosis appear to be independent events and do not associate together (Oh, McIntosh, & Schnitzer 1998). Newly synthesised cholesterol from the endoplasmic reticulum first appears at the caveolae before moving to the surrounding plasma membrane through currently unidentified mechanisms (Monier et al. 1996). The sequence responsible for cholesterol interaction has been mapped to tyrosine 14 and serine 80 although it should be noted that these two sequences differ in the mechanism of interaction with cholesterol (Fielding et al. 2004). Caveolae internalisation relies on cholesterol, as antagonism of cholesterol with progesterone reduces the internalisation of caveolae membranes (Smart et al. 1996). Caveolae have been implicated in oncogenic cell transformation, tumourigenesis and metastasis. This is based on evidence from cultured cells, animal models and human tumour tissue (Scherer et al. 1997; Bonuccelli et al. 2009).

The role of caveolae in signal transduction can be noted by caveolae orchestration and the co-localisations of several receptors as well as their subunits as shown in Table 1.1. Notably, caveolae house a plethora of canonical cardioprotective receptors, including the A_1AR and the δ-opioid GPCRs, as well as their G-protein subunits. The relationship between receptors and caveolae is dynamic, with receptors either moving in or out of caveolae upon activation. For example, under basal conditions, A_1AR is present in caveolae membranes but following A_1AR agonism using CCPA treatment, A_1AR relocates to the plasma membrane fractions as shown by co-immunoprecipitation and immunoelectron microscopy (Lasley et al. 2000). Activation of A_1AR is required for its translocation as A_1AR antagonism with DPCPX followed by agonism prevents the redistribution of A_1AR to plasma membrane fractions from caveolae (Lasley et al. 2000).

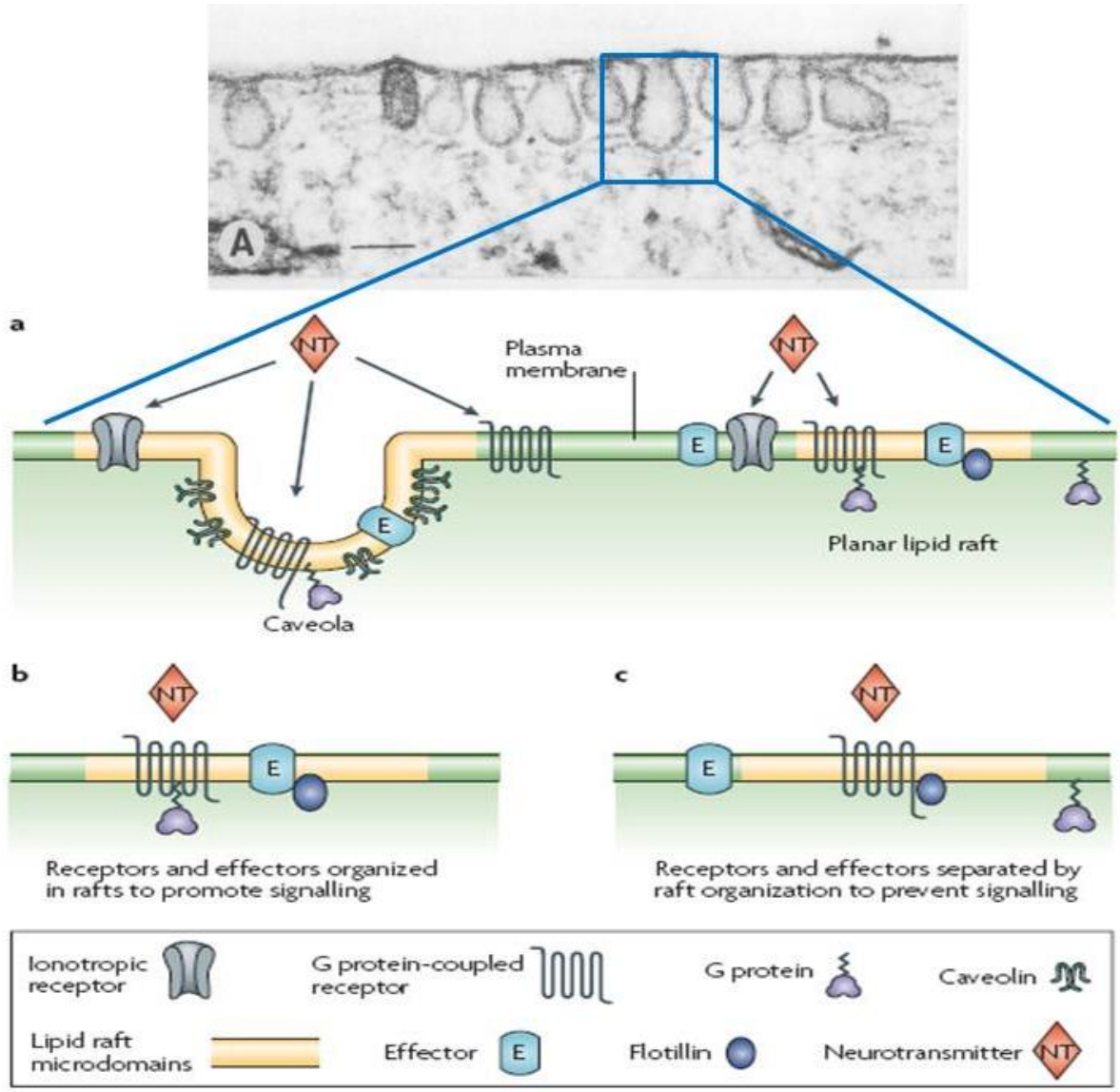


Figure 1.5 Caveolae are distinguished from the rest of the plasma membrane and lipid rafts by the presence of caveolin, cavin and Popeye-domain containing proteins which give it the unique flask-shaped morphology. It should be noted that caveolae may display varying morphology depending on the cell type when viewed under the electron microscope, with structures resembling grape-like clusters, rosette and tubular forms (above) as demonstrated in Figure 1 of Razani, Woodman & Lisanti (2002). In addition, caveolae compartmentalise GPCRs, their subunits and other type of receptors (non-GPCR) which has led to the formation of the caveolae signalling hypothesis. (Modified from Rothberg KG et al. 1992; Allen, Halverson-Tamboli & Rasenick 2007).

Table 1.1 Caveolae proteins localise and interact with many GPCRs, their subunits and receptor kinases. Modified from Razani, Woodman & Lisanti (2002a).

Caveolae Localisation	**Morphological Evidence**	**Biochemical Evidence**	**Interact with Caveolae proteins**
G-protein coupled receptors:			
A_1 adenosine receptor		+	+
Delta opioid receptor	+	+	
Bradykinin B2 receptor	+	+	
β1 and β2 adrenergic receptor	+	+	+
G-proteins:			
$G\alpha_s$	+	+	+
$G\alpha_{11}$		+	+
$G\alpha_{12}$		+	+
$G\alpha_0$			+
Gβγ		+	
$G\alpha_q$		+	
Receptor tyrosine kinases:			
Epidermal growth factor receptor			+
Non-receptor tyrosine kinases:			
Src		+	+
JAK2		+	
STAT3		+	
Non-receptor Ser/Thr kinases:			
Protein kinase A	+	+	+
Protein kinase C		+	
ERK	+		+
MEK			+
PI3-kinase		+	+

1.7.1 Caveolins

Caveolae can be distinguished from other members of the lipid raft family due to the expression of the caveolin family of scaffolding proteins. It has been estimated that each caveolae coat may have as much as 140-160 caveolin protein molecules (Pelkmans & Zerial 2005). The caveolin protein family consists of three members (Caveolin-1, -2 and -3), each encoded by a distinct gene (*CAV1*, *CAV2* and *CAV3*). The resulting proteins are structurally related, with all three containing an identical stretch of eight amino acids (FEDVIAEP) within their N-terminus end. The significance of this structural/functional motif is unknown, although it is highly conserved across species (Razani, Woodman & Lisanti 2002). *CAV1* and *CAV2* have been found to map to the 7q31 region of human chromosome 7 while *CAV3* maps to 3p25 (Engelman et al. 1998). Although *CAV1* and *CAV2* map upstream to tumour suppressor gene on human chromosome 7q31.1, the role of *CAV1* as a breast tumour suppressor gene has been refuted (Hurlstone et al. 1999). All three caveolin proteins display several isoforms and are similar in size (22-24 kD) and structure (Williams & Lisanti 2004). All caveolin proteins possess a scaffolding domain, oligomerisation domain, transmembrane domain and palmitoylation sites (Schegel, Pestell & Lisanti 2000).

The scaffolding domain is a negative regulator of variety of proteins involved in signalling such as G-protein subunits (G_{i2a}), eNOS, PKC isoforms and receptors such as EGFR (Razani, Woodman & Lisanti 2002). Compilation of proteins which interact with Cav1 and Cav3 scaffolding domain shows the interacting ligands almost invariably have the motifs Φ*X*ΦΦ, ΦΦΦ, Φ*X*ΦΦΦ, where Φ is an aromatic residue (Phe, Tyr, or Trp) and *X* is any amino acid (Couet et al. 1997). This ligand interaction does not occur with Cav2 scaffolding domain under the same conditions for Cav1 and Cav3 (Couet et al. 1997). Cav1 and Cav3 also possess the ability to form high molecular weight oligomers together ranging 200-400 kDs in size (Volonte et al. 2008). As shown by sucrose density gradients and confocal microscopy, caveolae have been shown to compartmentalise GPCRs and their subunits (Table 1.1) (Head et al. 2005). Confocal and electron microscopy demonstrates co-localisation of Cav3 and Gαs within the intercalated disks of cardiomyocytes and in the sarcolemma (Head et al. 2005). It has been suggested that G-protein subunits tether their receptors to caveolae. To support this,

decreased bradykinin$_2$ receptors and caveolae FRET measurements were observed when Gα_q-Caveolin-1 association was reduced by peptide inhibition (Calizo & Scarlata 2012).

It should be noted that caveolae, in addition to enhancing signalling, are also capable of inhibiting signalling. Disruption of caveolae via caveolin knockdown and cholesterol depletion has been shown to alter Gα_s trafficking in caveolae, prevent Gα_s internalization, and elevate Gα_s/AC activity in the mouse brain (Allen et al. 2009). The putative caveolin scaffolding domain (82-101) has been shown to inhibit many ligands including eNOS (García-Cardeña et al. 1997). All of these findings suggest that caveolae in conjunction with caveolins concentrate GPCR and their subunits to allow efficient signal transduction (Insel et al. 2005).

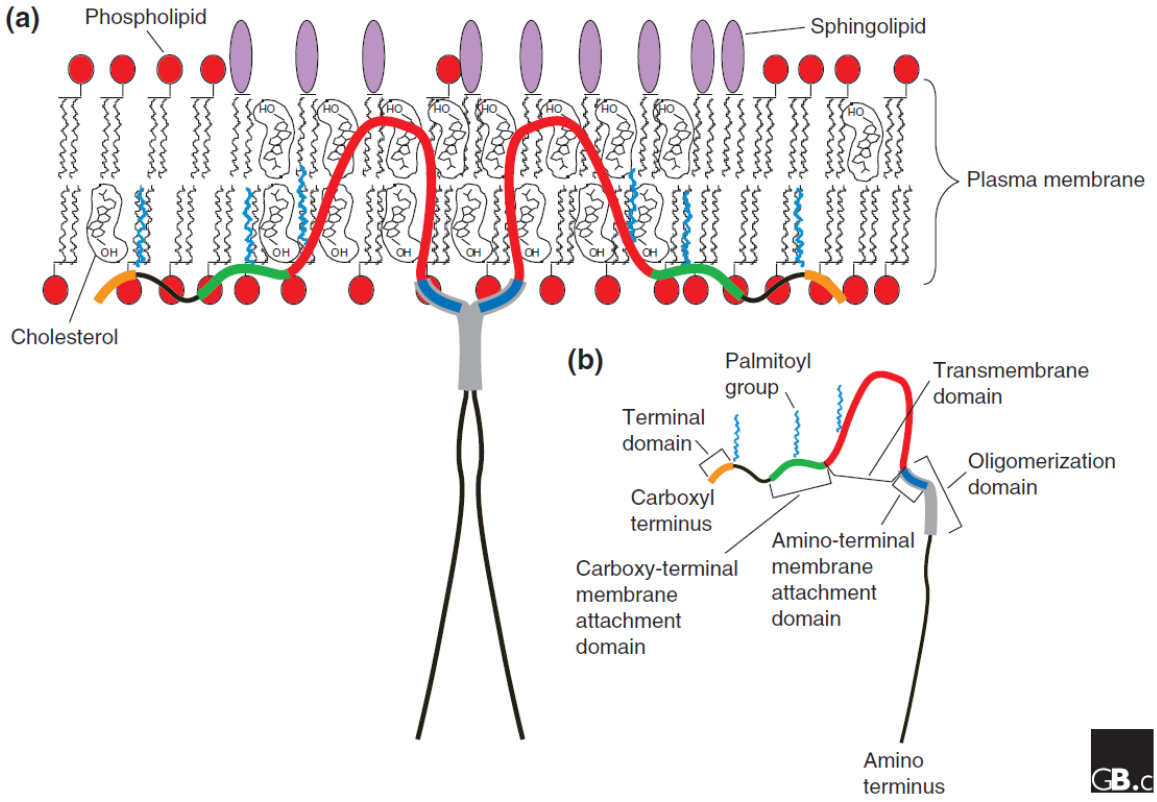


Figure 1.6 A**)** Caveolins are found in regions of the membrane that are particularly enriched in cholesterol and spingolipids. **B)** A single caveolin protein has many domains such as the oligomerisation and transmembrane domains that give caveolins proteins the ability to dimerise together and attach to the plasma membrane. For simplicity the caveolin oligomer is depicted as a dimer. Taken from Williams & Lisanti (2004).

1.7.1.1 Caveolin-1

Caveolin-1 was the first caveolae marker protein to be discovered (Rothberg et al. 1992). Cav1 displays 58% homology to Cav2 and 85% to Cav3 (Schlegel, Pestell & Lisanti 2000). On the human chromosome (7q31.1), *CAV1* and *CAV2* are in very close proximity to each other (≈19 kilobases apart) while *CAV3* is located on a different chromosome (3p25) (Williams & Lisanti 2004). Cav1 protein is ubiquitously expressed and is responsible for caveolae formation in non-muscle type cells such as endothelial, adipose and epithelial type tissue (Hansen *et al.* 2013; Drab et al. 2001). Two isoforms of Cav1 exist, the longer 178 amino acids long Cav1α and Cav1β which is truncated by 31 residues (Kogo & Fujimoto, 2000). There appears to be differences in forming caveolae efficacy between the Cav1 isoforms as differential introduction of Cav1 isoforms in HepG2 cells, which lack caveolae, show Cav1α is more efficient than the Cav1β isoform in forming caveolae (Fujimoto et al. 2000). Cav1 is believed to act as a tumour suppressor and a negative regulator of the EGFR-MAPK signalling cascade. In fact, Cav1 was first described as a protein that became tyrosine-phosphorylated (Tyr14) in Rous sarcoma virus transformed chick fibroblasts. The Tyr14 phosphorylation is believed to override the growth inhibitory activity of Cav1 (Li, Seitz & Lisanti 1996). Similarly, a *CAV1* mutation (P132L) observed in approximately 16% of human breast cancers is also believed to inactivate the same growth suppression, contributing to mammary cell invasiveness (Bonuccelli et al. 2009). Other notable *CAV1* mutations observed clinically was a single case of Berardinelli-Seip congenital lipodystrophy (BSCL), a rare recessive disease characterised by near absence of adipose tissue, and for a case of hypertriglyceridemia (Kim et al. 2008; Cao et al. 2008).

The absence of detectable Cav1 protein expression in common cancer cell lines including HeLa, Jurkat and 3T3 L1 cells further supports its role as a tumour suppressor (Scherer et al. 1997). Consistent with these findings, the reintroduction of the Cav1 gene into Cav1 deficient NIH 3T3 cells abrogates abnormal cell growth and even causes apoptotic cell death (Engelman et al. 1997). For further summary please refer to Williams & Lisanti (2004). Cav1-deficient mice show dramatic reduction (≈50%) in life span when compared to wild-type mice (Park et al. 2003). Phenotypically, Cav1-deficient mice show a combination of pulmonary fibrosis, pulmonary hypertension, and cardiac hypertrophy (Park et al. 2003). Cardiac hypertrophy

observed in Cav1-deficient mice is thought to arise from the loss of Cav1 inhibiting Erk1/2 kinase pathways in cardiac fibroblasts and myocytes (Galbiati et al. 1998; Page & Doubell 1996). As shown by electron micrography, double Cav1/Cav3-deficient mice show caveolae disappear in cardiomyocytes and in the adjacent endothelial cells while Cav3-deficient mice show loss of caveolae in the cardiomyocyte but not in the adjacent endothelial cell (Park et al. 2002). Whilst Cav1-deficient animals show disappearance of caveolae in the endothelial cells but not in cardiomyocytes (Park et al. 2002). Although not widely associated with cardioprotection, Cav1 has been shown to be required for cardioprotective effects of methods such as IPC and anaesthetics preconditioning with infusion of Cav1 peptide shown to be cardioprotection as well (Das et al. 2012; Patel et al. 2007; Tsutsumi et al. 2010; Young, Ikeda & Lefer 2001; Shen et al. 2011). The mechanisms of this protection has been shown to involve Src-mediated phosphorylation of Cav1 and eNOS release as antagonism of Src and eNOS shown to reverse the cardioprotective effects of Cav1 peptide infusion (Patel et al. 2007; Young, Ikeda & Allan 2001). Similarly, in MDCK cells phospho-Cav1 has been shown to promote cell survival following oxidative stress although its molecular mechanisms remain uncharacterized (Percy et al. 2008). Growth factors such EGF and insulin which activate the cardioprotective receptors EGFR and IGFR (respectively) have been shown to result in significant increase of Cav1 phosphorylation (Kim et al. 2000; Kimura et al. 2002). The internalization of the cardioprotective tyrosine kinase receptor such as insulin-like growth Factor-I receptor (IGF-IR) and EGFR also requires phospho-Cav1 although this has yet to be shown in cardiomyocytes (Salani et al. 2010; Monaghan-Benson, Mastick & McKeown 2008).

Although it has yet to be shown in cardiomyocytes, Cav1 is crucial for mechanoprotection and stress resistance in several types of cells. In 3T3 fibroblasts phosphorylation of Cav1 has been shown to be required for Dynamin1-mediated internalization of caveolae, with internalization of caveolae thought to be a crucial step in membrane repair (del Pozo et al. 2005; Corrette et al. 2013). Dynamin-dependent endocytosis has been shown to be crucial component of plasma membrane repair following membrane injury in T lymphocytes and in endothelial cells (Kang-Decker et al., 2007; Bernatchez et al. 2009). Similarly, endothelial caveolae have been shown to protect cells from membrane damage during increases in cardiac output and hypoxia in vivo as Cav1-deficiency shown to result in decreased membrane integrity following increased cardiac output and hypoxia (Cheng et al. 2015). Cav1 is also implicated in autophagy

in endothelial cells as Cav1 knockdown (siRNA) has been shown to result in increased autophagy and oxidative stress (Shiroto et al. 2014). Despite the growing evidence that Cav1 is a prerequisite to variety of cellular functions (i.e. autophagy, membrane repair) as mentioned previously, the exact mechanisms of Cav1-mediated cardioprotection remain largely uncharacterised, perhaps owing to the popularity of Cav3.

1.7.1.2 Caveolin-2

The discovery of Caveolin-2 led to the formation of caveolin family of genes (Scherer et al. 1996). Cav2 has 59% homology to Cav1 and 60% homology to Cav3 (Schlegel, Pestell & Lisanti 2000). It is hypothesised that Cav2 is the genomic precursor of Cav1 and Cav3, which are thought to be products of the fusion of the exon 2 in *Cav2* (Williams & Lisanti 2004). Cav2 expression parallels that of Cav1. Three isoforms of Cav2 have been identified to date, Cav2α, Cav2β and Cav2γ (Razani et al. 2002a). Their roles in the cell and in forming caveolae are unknown. However, they are differentially expressed in lung, adipose and heart tissue with the heart expressing Cav2γ (Razani et al. 2002b). Cav2-deficient animals do not show perturbations of Cav1 levels in the fat and lung issue whilst heart tissue show reduced Cav1 (Razani et al. 2002b). This decrease in Cav1 expression may be due to decreased Cav2 targeting of Cav1 to membrane, which forms heterooligomers together targeting to the plasma membrane and caveolae (Lahtinen et al. 2003). Skeletal muscles of Cav2-deficient mice show tubular aggregate formation, and display delayed skeletal muscle regeneration following injury (Schubert et al. 2007). Although not essential for the formation of caveolae, phosphorylation of Cav2 appears vital for attachment of Cav1-dependant caveolae as mutations of a single Cav2 phosphorylation residue greatly reduces attachment of caveolae to the plasma membrane, whilst mutations in two residues results in ablation of caveolae (Sowa et al. 2003). Cav3 levels are not perturbed in Cav2-deficient mice, which retain caveolae in muscle type cells (Razani et al. 2002b; Schubert et al. 2007). Cav2-deficient animals show significantly reduced tolerance to exercise which may be attributed to lung abnormalities observed in Cav2-deficient phenotype (Razani et al. 2002b).

1.7.1.3 Caveolin-3

Unlike Cav1 and Cav2, Cav3 has not been shown to produce multiple isotypes. Cav3 displays 85% similarity to Cav1 whilst this is 60% to Cav2 (Schlegel, Pestell & Lisanti 2000). Cav3 expression is limited to muscle-type tissue with skeletal muscle and cardiomyocytes shown to have high expression of Cav3 (Liu et al. 2008). The importance of Cav3 in these tissues can be highlighted by existence of "caveolinopathies" with to date 30 known mutations in *CAV3* gene, which result in variety of clinical phenotypes (Gazzerro et al. 2010). Briefly, mutations in the *CAV3* gene results in a variety of muscle and cardiac disorders including Limb Girdle Muscular Dystrophy 1-C, idiopathic persistent elevation of serum creatine kinase, inherited rippling muscle disease, distal myopathy and familial hypertrophic cardiomyopathy and some cases of sudden infant death syndrome (Gazzerro et al. 2010; Cronk et al. 2007).

Although the Cav3 protein is a relatively small peptide composed of 151 amino acids, it displays several domains which assist Cav3 in membrane attachment, oligmerasation and protein interactions. Most notably the caveolin scaffolding domain which is conserved in both Cav1 and Cav3 (residues 82-101) contains the signature motif which typically bind to other motifs containing ϕXϕϕ, ϕϕϕ or ϕXϕϕϕ, where ϕ is an aromatic and X an unspecified amino acid (Byrne, Dart & Rigden 2012). The caveolin scaffolding domain has been shown to interact with GPCRs such as A_1AR, RTKs such as EGFR and other cardioprotective proteins including eNOS (Roth & Patel 2011). The delivery of a modified Cav3 peptide composing of only the scaffolding domain (82-101) is cardioprotective (Young et al. 2001).

Cardioprotective stimulus such as IPC is known to increase caveolae formation. IPC-mediated protection can be abolished by disruption of caveolae through cholesterol depletion via methly-β-cyclodextrin treatment (Horikawa et al. 2008). Cholesterol depletion has been shown to abrogate the cardioprotective benefits of IPC, non-GPCR and GPCR induced preconditioning thus highlighting caveolae as important signalling centres (Horikawa et al. 2008; Tsutsumi et al. 2010). As previously discussed, Cav3 is expressed in cardiomyocytes and is responsible for caveolae formation in cardiomyocytes (Park et al. 2002). Caveolae in cardiomyocytes has been shown to host numerous GPCRs as well as their subunits (Head et al. 2005). Under basal conditions, Cav3 is particularly enriched in fractions 4-5 and to a lesser degree in 6-12 (Head et al. 2005). Ischaemia causes re-localisation of Cav3; 90% of Cav3 immunoreactivity was found in fractions 4–7 in normal non-ischaemic cells while it was found

to be 70% of fractions 4-7 in ischaemic and non-ischaemic zones (Ballard-Croft et al. 2006). As caveolae membranes are primarily enriched in fractions 4-7, no change in total protein expression accompanied by a shift in fraction migration may represent a different localisation within the plasma membrane.

Caveolae have been shown to localise GPCR such as A_1AR and the δ-opioid receptor which are key cardioprotective receptors (see Table 1.1) (Patel et al. 2006; Lasley & Smart 2001). Under basal conditions, A_1AR is present in Cav3 caveolae membranes, following A_1AR agonism using CCPA treatment A_1AR relocates to other plasma membrane fractions as shown by co-immunoprecipitation and immunoelectron microscopy (Lasley et al. 2000). A_1AR antagonism with DPCPX followed by agonism prevents the redistribution of A_1AR, suggesting activation may be necessary to redistribute A_1AR to plasma membrane fractions from caveolae (Lasley et al. 2000). CCPA agonism results in increased translocation of PKCδ and PKCε into Cav3 caveolae as evidenced by immunoprecipitation and confocal microscopy (Yang, Sung & Hu 2009). CCPA treatment co-immunoprecipitates eNOS with Cav3, which suggests eNOS may be a target of A_1AR activation (Lasley et al. 2000). The presence of A_1AR and adenosine deaminase within internalising vesicles "caveosomes" has been shown at certain occasions although their return back to cell membrane differed as adenosine deaminase recycles toward caveolae-like structures, whereas A_1AR recycles to non-caveolae membrane regions (Eschriche et al. 2003). It is currently unclear whether A_{2A}AR, A_{2B}AR and A_3AR play a role in caveolae-mediated signalling although they do express caveolin-binding motifs and therefore may potentially localise or interact with residents of caveolae and the caveolin proteins. The C-terminus of A_1AR has been shown to interact with Cav1 via the caveolin binding motifs (Eschriche et al. 2003).

Immunohistochemistry staining and co-immunoprecipitation demonstrates co-localisation of δ-opioid receptor and Cav3 at the plasma membrane and in a transverse pattern along the length of the cardiomyocyte (Patel et al. 2006). The extent of this co-localisation has been estimated to be approximately 78% (Patel et al. 2006). δ-opioid receptor agonism (by SNC-121) results in a reduction in cell death but when combined with cholesterol depletion (by MβCD) this reduction in cell death is abrogated (Patel et al. 2006). This implicates that δ-opioid receptor-mediated cardioprotection is reliant on caveolae. The B_2 bradykinin receptor is another cardioprotective receptor found in caveolae. Prior to stimulation, B_2 bradykinin

receptors display a random distribution within the plasma membrane (Haasemann et al. 1998). Beyond the fact that Cav3 and B_2 bradykinin receptors co-localise with eNOS little is currently known regarding the relationship of Cav3 and this receptor in cardiomyocytes (Quinlan et al. 2008). However, in A431 epidermoid carcinoma cells, B_2 receptor agonism results in co-localisation with Cav1, in contrast to A_1AR agonism, which causes A_1AR to relocate to other plasma membrane fractions from the caveolae membrane (Haasemann et al. 1998).

Cav3 co-localises with eNOS in the heart although it must be noted that this localisation of eNOS is not exclusive to caveolae as eNOS is also present in non-caveolae fractions (Massion et al. 2004). In addition, Cav3 does not positively regulate activity of eNOS as well as other NOS isoform in the heart as Cav3-overexpression does not result in increased NOS activity in the heart (Tsutsumi et al. 2008). Electron microscopy has identified caveolae adjacent to mitochondria in subsarcolemmal and interfibrillary mitochondria, which express Cav3 mostly in the inner mitochondrial matrix (Fridolfsson et al. 2012). IPC and sub lethal ischaemia results in movement of sarcolemmal caveolae into the mitochondria with this trafficking shown to be dependent on Gi protein (Wang et al. 2014). *Cav3* overexpression results in enhanced resistance to calcium tolerance, greater respiratory rate during I-R and reduced ROS production following hypoxia-reoxygenation when compared to wild-types (Fridolfsson et al. 2012). As a result, marked preservation of the mitochondria in the Cav3 overexpressing hearts may contribute to enhanced recovery of these hearts (Fridolfsson et al. 2012). The molecular effectors of this interaction are beginning to be unravelled. Mitochondrial proteins have been shown to be targets of caveolae-derived eNOS S-nitroyslation as Cav3-deficiency and MβCD treatment results in loss of IPC-mediated S-nitroyslation of mitochondrial preparations (Sun et al. 2012; Sun et al. 2015). Surprisingly, Cav3-overexpression does not increase the expression of the NOS isoforms, which localise in caveolae fractions, with NOS activity and translocation in the membrane was also unaltered (Tsutsumi et al. 2008).

1.7.2 Cavin Proteins

Recently, a new family of caveolae proteins termed cavins (Cavin1, 2, 3 and 4) have been re-classified for their roles in caveolae biogenesis and localisation. These four cavin proteins share varying degrees of homology relative to each other with all cavin proteins displaying two PEST (proline, glutamic acid, serine and threonine-rich domains) as a signature motif (Briand, Dugail & Le Lay 2011). It is not known what function PEST domains have in the cavin proteins, it is known that PEST domains can promote protein degradation through μ-calpain and 26S proteasome system (Shumway, Maki & Miyamoto 1999; Revert et al. 2001). All cavin proteins also share a conserved coiled-coil domain and a membrane association domain (Parton & del Pazo 2013). Unlike CAV1 and CAV2 which map on the same chromosome, all cavins map to distinct regions in the human chromosome, CAVİN1 maps to 17q21.2, CAVİN2 to 2q32.3, CAVİN3 to 11p15.4 and CAVİN4 to 9q31.1. Using sequence homology search, the human and mouse Cavin1 protein shares strong homology (93% identical) as do the human and mouse Cavin2 (83%), Cavin3 (78%) and Cavin4 (89%) homologs. All cavin proteins are larger in size when compared to the caveolins; Cavin1 is 390 amino acids long, Cavin2 425 amino acids, Cavin3 261 amino acids and Cavin4 364 amino acids in size (Parton & del Pozo 2013). Whilst the caveolin proteins function as coat proteins within the caveolae, the cavins are thought to function as support or accessory proteins and in some cell types crucial for caveolae formation as discussed in their respective sections. Similar to caveolins, all members of the cavin family possess one or more phosphorylation sites, although it is unknown what impact this has on their functions (Hansen & Nichols 2010). The expression of cavin proteins has been shown to be uniform around the caveolae bulb as shown by immunogold labelling (Ludwig et al. 2013). Single-molecule fluorescence measurements suggest that cavin monomers consist of 50 molecules and have a limit of formation perhaps imposed by the size of the caveolae curvature (Gambin et al. 2014). Interestingly, caveolae regions with cavin proteins do not assemble under the same complexes, with Cavin1 subcomplexes not having Cavin2 and Cavin3 at the same time but rather containing distinct Cavin1-Cavin2 and Cavin1-Cavin3 complexes (Gambin et al. 2014).

Table 1.2 Revised nomenclature for the cavin family of proteins. (Modified from Bastiani et al. 2009).

Old Nomenclature	New Proposed Nomenclature
Polymerase I and transcript factor (PTRF) Cav-p60 Cavin	Cavin1
Serum deprivation response protein (SDPR) Phosphatidylserine-binding protein	Cavin2
Protein kinase C delta binding protein (PKRCBP) Serum deprivation response factor-related gene product that binds to C-kinase (SRBC)	Cavin3
Muscle-restricted coiled-coil protein (MURC)	Cavin4

1.7.2.1 Cavin1

Cavin1 was initially described as a Polymerase I and transcript release factor (*PTRF*) as the dissociation of transcripts from Polymerase I and its tertiary structure required the presence of this protein (Jansa et al. 1998). It was later reclassified as it was particularly enriched caveolae fractions and immunogold labelling showed distinct caveolae localisation (Vinten et al. 2005). In terms of homology, Cavin1 is 66% to Cavin2, 59% to Cavin3 and Cavin4 (Bastiani *et al*. 2009). Cavin1 is moderately expressed in the heart with adipose and lung tissue also showing notable expression of Cavin1 (Liu et al. 2008). In the heart, Cavin1 is enriched in sucrose density fractions 4-12 whilst Cav3 is enriched in fractions 11-12 suggesting there may be distinct subcomplexes (See Table 1.3) (Hansen et al. 2013; Tsutsumi et al. 2008). However, IPC causes increased translocation of Cav3 to fractions 4-5 which are particularly enriched in Cavin1 protein (Hansen et al. 20130; Tsutsumi et al. 2008). Cavin1 localises with Cav3 and is essential for caveolae formation in some cell types while also simultaneously acting as an accessory protein (Liu et al. 2008; Hansen et al. 2013). Cavin1-deficiency results in loss of caveolae in lung epithelium, intestinal smooth muscle, endothelial cells, skeletal muscle and cardiomyocytes (Liu et al. 2008; Kasahara et al. 2014). Interestingly, caveolin mRNA is up-regulated in Cavin1-deficient tissue although at the proteomic level there is a reduction in

expression compared to their wild-type counterparts (Liu et al. 2088). Cavin1 has been shown to be involved in stability of Cav1 and Cav3 as knockdown of Cavin1 results in caveolins to diffuse freely in the plasma membrane which eventually becomes internalized into the endolysosomal system for degradation (Hill et al. 2008). Similarly, rats with heart failure show significant Cavin1 down-regulation accompanied by Cav1 and Cav3 down-regulation, suggesting Cavin1 may stabilise caveolins (Norman et al. 2014). The exact role of Cavin1 in cardioprotection is unknown, however Cavin1 has been shown to interact with the plasma membrane repair proteins such as Mg53 and Smpd1 (Zhu et al. 2011; Corrette et al. 2013). Loss of Cavin1 via mutations or knockout approaches show a phenotype of generalised lipodystrophy, glucose intolerance, cardiodystrophy and muscular dystrophy (Hayashi et al. 2009; Liu et al. 2008; Kasahara et al. 2014).

1.7.2.2 Cavin2

Cavin2 is also known as serum deprivation response protein (Sdpr) was discovered in the early 1990s as an mRNA that was highly induced in serum starved 3T3 fibroblasts (Gustincich & Schneider 1993). Its relation to caveolae was discovered in the late 1990s as a protein that binds PKCα to caveolae (Mineo et al. 1998). Cavin2 is expressed abundantly in the heart with lung and fat tissue also showing notable Cavin2 expression (Hansen et al. 2013). It is currently unknown whether Cavin2 has isoforms. Cavin2 protein is 66% identical to Cavin1, 68% to Cavin3 and 57% to Cavin4 (Bastiani et al. 2009). In the heart, Cavin2 mainly fractionates in 5-9, whilst Cav1 and Cav3 mainly in 11 and 12 (Hansen et al. 20130; Tsutsumi et al. 2008). Unlike Cavin1, Cavin2-deficient animals do not show any perturbations in caveolin and cavin expression in the heart. Although not essential for caveolae formation in some cells, Cavin2 is required for caveolae formation in lung endothelium but not in heart endothelium (Hansen et al. 2013). Similar to Cav2, Cavin2 may act as an accessory protein by controlling caveolae depth and targeting Cavin1 to caveolae as Cavin2 knockdown has been shown to lead to retention of Cavin1 in the cytoplasm and decrease in the membrane fraction in 3T3 adipocytes (Breen et al. 2012). To support its role as an accessory protein, unlike overexpression of Cavin1, Cavin2 overexpression results in deformation of caveolae and extensive tubulation of the plasma membrane (Hansen et al. 2009). In terms of cardioprotection Cavin2 remains largely uncharacterised. However, it is possible that Cavin2

is a negative regulator of Akt and Erk1/2 signalling as Cavin2-deficient hearts display hypertrophy and are resistant to apoptosis induced by hypoxia and H_2O_2 stimulation (Maruyama et al. 2014).

1.7.2.3 Cavin3

Similar to Cavin2, Cavin3 was initially identified as a PKC binding protein although it was later associated with caveolins and caveolae (McMahon et al. 2009). Cavin3 protein is moderately similar to Cavin1 (59%), Cavin2 (68%) and Cavin4 (51%) (Bastiani et al. 2009). Cavin3 is expressed at comparable levels to the caveolins in the heart (Hansen et al. 2013). Alternative splicing of *Cavin3* mRNA can yield up to five isoforms with sizes ranging from 14 to 31-kDa (McHahon et al. 2009). Cavin3 is expressed in variety of cell types such as adipocytes, brain and is interestingly expressed highest in the heart according to proteomic studies by others (Bastiani et al. 2009). In the heart, Cavin3 mainly co-fractionates in fractions 3-10 with small fractionation in 11-12, whilst Cav1 and Cav3 are observed mostly in 11 and 12 (Hansen et al. 20130; Tsutsumi et al. 2008). Cavin3 appears important in cell signalling as Cavin3 can regulate Erk, Akt and EGFR signalling and is thought to couple these signalling components to intracellular transport machinery (Hernandez et al. 2013; McMahon et al. 2009). Cavin3 may couple caveolae signalling through budding of vesicles from caveolae termed “cavicles” using microtubules as absence of Cavin3 reduces cavicle trafficking (McMahon et al. 2009). Furthermore, recent studies suggest the existence of two caveolae types, caveolae that are stably associated with the cell surface and “kiss-and-run” type of caveolae which undergo rounds of fission and fusion (Mohan et al. 2015). The contents of these cavicles are unknown and should be investigated. Lung tissue deficient of Cavin3 show reduced phospho-Erk levels and increased phospho-Akt levels when compared to wild-type (Hernandez et al. 2013). Cavin3-deficieny in the heart does not alter caveolin and cavin expression (Hansen *et al.* 2013). Pleiotropic phenotype is seen in Cavin3-deficient animals, most notably shortened life span and late onset cachexia (Hernandez et al. 2013). Cavin3-deficient hearts show differential expression in a variety of genes notably caveolae transcripts such as ≈1-fold increase in Cav1-2 and Cavin1-2 (Liu et al. 2014). The role of Cavin3 remains uncharacterised in cardioprotection.

1.7.2.4 Cavin4

Cavin4 is the most recent addition to the cavin family. In both humans and mouse, Cavin4 is encoded by two exons that are conserved in both species yielding a Cavin4 protein that is 362 amino acids long (Bastiani et al. 2009). Currently there is no sucrose density fractionation data available for Cavin4, although it is unknown Cav3 and Cavin4 co-localise together in the sarcolemma of myoblasts (Bastiani et al. 2009). Cavin4 has no known splice variants. Unlike the other cavins, Cavin4 is a muscle specific protein expressed only in heart and muscle tissue (Bastiani et al. 2009). Cavin4 is thought to not be essential for caveolae formation although it can influence caveolae morphology as Cavin4-overexpression results in increased size of caveolae compared mock-treated cells (Ogata et al. 2014). Maturation of cardiomyocytes and cultured myocytes involves up-regulation of Cavin4 with expression of Cavin4 being shown to be perturbed in myopathies along with Cav3 (Ogata et al. 2008; Bastiani et al. 2009). Cavin4 localises in caveolae and T-tubules of cardiomyocytes and also localises phospho-Erk 1/2 to caveolae as shown by immunoelectron labelling (Ogata et al. 2012). Cavin4 together with Cav3 co-localises with α1-adrenoceptor which is dependent on Cav3 as knockdown of Cav3 does not affect the trafficking and localisation of α1-adrenoceptor, whilst Cavin4 accumulates in the cytosol (Ogata et al. 2014). Cavin4 overexpression in mice hearts has been shown to induce fibrosis, ventricular hypertrophy, atrial arrhythmias, and changes in gene expression including contractile-related transcripts such as Mhy7 and Serca (Ogata et al. 2008). Conversely, Cavin4-deficient hearts show reduced PE-induced hypertrophy when compared to wild-types (Ogata et al. 2012). Similar phenotypic correlations were made in a large human study, which identified six mutations in the human *CAVİN4* gene which resulted in hypertrophic cardiomyopathy (Rodriguez et al. 2011). Hypoxia has been shown to increase Cavin4 expression in rat cardiomyocytes, which has been attributed to increased hypoxia-induced Cavin4 promoter activity mediated by the DNA binding protein Serum Response Factor (Shyu et al. 2014).

1.7.3 Popdc Proteins

Although not widely associated with caveolae, members of the Popeye domain-containing (*POPDC*) gene family have also shown to be crucial for caveolae formation and caveolae-related functions (Alcalay et al. 2013). The POPDC family consists of three members: *POPDC1, POPDC2* and *POPDC3*. In humans *POPDC1* and *POPDC3* genes are organized in tandem on chromosome 6q21 while POPDC2 is located on chromosome 3q13.33 (Shindler et al. 2012). As *POPDC2* is uniquely present in vertebrates, it is hypothesised that *POPDC2* appeared by gene duplication from *POPDC3* (Shindler et al. 2012). Popdc2 and Popdc3 display 50% sequence identity on the protein level, while Popdc1 is only 25% similar to Popdc2 and Popdc3 (Andree et al. 2002). Popdc expression is restricted to muscle-type cells although some epithelial cells also express Popdcs such as those found central and autonomic nervous system and in epithelial cells of the epidermis, gastrointestinal tract, retina, lens, and cornea (Schindler et al. 2012). Popdcs are ≈43-kDa transmembrane proteins that contain the two highly conserved sequence motifs (FL/IDSPEW/F and FQVT/SL/I) crucial for cAMP binding (Brand et al. 2014). The amino terminus of Popdc can be N-glycosylated, which is responsible for the apparent molecular mass of 58-kDa of Popdc1 on PAGE gels although it has a predicted mass of 43-kDa (Andree et al. 2000).

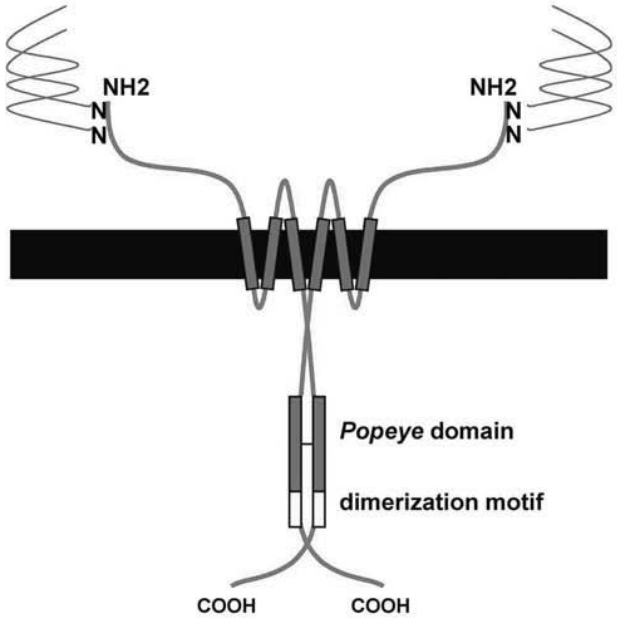


Figure 1.7 Proposed model for Popdc proteins which contain a transmembrane domain that spans the plasma membrane three times, cytoplasmic domain which contains the Popeye domain and dimerization domain. (Taken from Brand 2005).

Popdc1 (a.k.a. Blood vessel/epicardial substance) is predicted to have two isoforms, *Popdc2* with five, whilst the existence of *Popdc3* isoforms are uncharacterised (Breher et al. 2004). *Popdc1* is 359 amino acids long, *Popdc2* is 367 amino acids and *Popdc3* is 292 amino acids (Andree et al. 2000). Similar to caveolins, Popdcs can homodimerise together in complexes 97-212 kDa in size (Knight, Bader & Backstrom 2003). Dimerisation of Popdc1 involves lysines 272 and 273 at the carboxylic terminal (Kawaguchi et al. 2008). Currently, very little is known about the exact physiological function of Popdcs. They have been implicated in variety of processes such as chronotropic response, cell regeneration and regulation of membrane processes (Froese et al. 2012; Hager et al. 2010; Alcalay et al. 2013). In the heart Popdcs are thought to maintain cardiac pace making as *Popdc1* and *Popdc2* disruption results in stress-induced sinus node dysfunction, resulting in chronotropic incompetence and long sinus pauses (Froese et al. 2012). It is thought that the arrhythmias associated with Popdc disruption is due to loss of TREK-1 potassium channel activity, which co-localises with Popdc1 and Popdc2 (Froese et al. 2012). Popdcs are also involved in membrane processes such as membrane trafficking and caveolae formation. Popdc1 co-immunoprecipitates with VAMP3, which is a component VAMP-SNARE complex involved in the docking and/or fusion of caveolae with the target membrane (Hager et al. 2010; Mehta & Malik 2006). VAMPs are required for caveolae-mediated trafficking as VAMP inhibition results in accumulation of caveolae ligands as well as reduced export of natriuretic peptide (McIntosh & Schnitzer 1999; Ferlito et al. 2010). Popdc1 has been shown to influence caveolae formation as Popdc1-deficient cardiomyocytes display reduced size and number of caveolae (Alcalay et al. 2013). To date, whether there is any role of Popdc2 and Podpc3 in caveolae biogenesis remain uncharacterised.

				BF					non-BF		
Fraction #	1	2	3	4	5	6	7	8	9	10	11	12
Cav1					+	+	+	+	+	+	+	+
Cav2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Cav3										+	+	+
Cavin1			+	+	+	+	+	+	+	+	+	+
Cavin2					+	+	+	+	+			
Cavin3			+	+	+	+	+	+	+	+	+	
Cavin4	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Popdc1					+	+	+	+	+	+	+	+
Popdc2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Popdc3	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Table 1.3 Sucrose density sedimentation of caveolins, cavins and Popdcs. Compiled from Tsutsumi et al. (2008); Hansen et al. (2013); Alcalay et al. (2013). BF= Buoyant Fraction, non-BF= Non-Buoyant Fraction, nd = not determined.

1.8 Hypothesis & Aims

As previously discussed, caveolae and its coat proteins have been shown to be required for cardioprotective effects of ischaemic and pharmacological preconditioning. However, the aged heart displays refractoriness to these protective stimuli with the expression of caveolar coat proteins being poorly characterised in the aged hearts, which may partially be responsible for the loss of the efficacy of these stimuli in these hearts. Furthermore, there are sex-related differences in cardioprotection as shown by epidemiological and experimental studies, which implicates sex-related differences in Cav3. The age and sex-dependant expression of caveolins, cavins and Popdcs remain largely uncharacterised which as discussed previously contribute to caveolar biogenesis and signalling. For the ageing model, we employed a time-course analysis of 8-, 16-, 32- and 48-week old normoxic and post-ischaemic hearts which have previously been shown to display age-related loss of cardioprotection at 48-weeks whilst most studies have shown this for >74-week old hearts (Willems et al. 2006). The objectives of this dissertation were to elucidate the expression of caveolae structural protein caveolins, cavins and Popdcs in cardiac ageing. Thus the expressions of caveolins, cavins and Popdc transcripts in the ageing male and female hearts was analysed in Chapters 3 and 4, respectively.

The objective of Chapter 5 was to further investigate the involvement of Cav3, major structural protein of caveolae, in the involvement of I-R tolerance using *in vitro* methods. The objective of Chapter 6 was to elucidate the expression of several candidate miRNAs involved in ageing/senescence and I-R as miRNA have been shown to display differential expression with ageing and I-R. Furthermore, using bioinformatics prediction, miRNA that were predicted to target the crucial caveolae forming transcripts Cav3 and Cavin1 were analysed in the ageing male normoxic and post-ischaemic hearts. The objectives of Chapter 7 were to investigate the expression of several plasma membrane repair proteins and proteases which remain largely uncharacterised in the ageing male heart. Plasma membrane-repair mechanisms have been shown to be reliant on caveolae and their coat proteins for normal trafficking and targeting to sites of injury. Chapter 8 presents an overall summary of the key findings within the dissertations, including the implication of the results and future directions to better understand the role of caveolae coat proteins.

2. Chapter Two

Materials and Methods

2.1 Samples

2.1.1 Perfused mouse heart model

All hearts used in this study were obtained from a cardiac biobank established from male and female wild-type C57BL/6 mice. The collection of these hearts were previously approved by and performed in accordance with the guidelines of the Animal Ethics Committee of Griffith University, which is accredited by the Queensland Government, Department of Primary Industries and Fisheries under the guidelines of "The Animal Care and Protection Act 2001, Section 757". Hearts were acquired from male and female C57BL/6 mice aged 8 (young), 16 (young-mature), 32 (adult) and 48-week old (middle-aged) (n=8-12/group). We chose these timepoints as Willems et al. (2005) has shown significant sex and age-dependant changes in oncosis and contractile dysfunction are present in these time points well before the aged (74-week old) phenotype. This type of ageing model has been adopted by other investigators to understand pathophysiology of variety of diseases including I-R and have a high degree of relevance to humans (Vanhooren et al. Libert 2013; Willems et al. 2005). Despite these significant changes, the molecular basis of I-R injury remains largely uncharacterised for both males and females.

Mice were anesthetized with 60 mg/kg sodium pentobarbital and hearts excised. The details of the isolated heart perfusion model and the I-R protocols employed are outlined in detail in prior works (Headrick et al. 2003; Peart et al. 2007; Willems et al. 2006). Briefly, hearts were subjected to normoxic perfusion or 20 minutes of global normothermic ischaemia and 60 minutes of reperfusion. All hearts were immediately stored in RNA*later*® solution (Life Technologies, Carlsbad, CA, USA) and stored at 4°C overnight. For long-term biobanking, hearts were transferred to -80°C storage to protect RNA integrity and expression levels prior to ventricular dissection and RNA and protein extraction. Coronary venous effluent was sampled during the 60-minute post-ischaemic period. Effluent was stored at 4°C and assayed the following day for lactate dehydrogenase (LDH) activity using the Cytotox 96® Non-radioactive Cytotoxicity Assay (Promega, Madison, WI, USA).

2.1.2 HL-1 cardiomyocyte cell culture

Cells of the murine atrial-derived cardiac cell line HL-1 were a kind gift from Professor William C. Claycomb (Louisiana State University, Baton Rouge, LA). Cells were grown in culture vessels pre-coated with 0.00125% fibronectin in 0.02% gelatin and maintained in Claycomb medium (Sigma Aldrich, St. Louise, MO, USA) supplemented with 10% foetal bovine serum (Life Technologies, Carlsbad, CA, USA), 0.1 mM norepinephrine, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, and 0.25 µg/mL amphotericin B. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Media was replaced every second day and cells passaged at >80% confluency when required using 0.25% Trypsin-EDTA (Life Technologies). For transfection experiments, cells were passaged during logarithmic growth (~50%). Cells were screened every two months for mycoplasma contamination using the MycoAlert™ Mycoplasma Detection Kit (Lonza, Basel, Switzerland). Passaged cells were continually monitored for viability using Countess cell counting slides (Thermo-Fisher, Waltham, MA, USA)).

2.1.3 3T3 fibroblast cell culture

3T3 mouse embryo fibroblasts (CRL-1658) were obtained from the ATCC (Manassas, VA, USA) and were grown in fully supplemented DMEM with 10% foetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, and 0.25 µg/ amphotericin B (Life Technologies). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Media was replaced every second day and cells passaged when required using 0.25% Trypsin-EDTA (Life Technologies). Cells were screened every two months for mycoplasma contamination using the MycoAlert™ Mycoplasma Detection Kit (Lonza, Basel, Switzerland). Passaged cells were continually monitored for viability using Countess cell counting slides (Thermo-Fisher).

2.2 RNA isolation and cDNA synthesis

2.2.1 RNA isolation from left ventricular tissue

RNA*later*® stabilised whole hearts were removed from -80°C storage and allowed to thaw on ice. Atrial and vascular tissue was removed with a scalpel and left ventricular myocardium dissected from each heart. Each left ventricle was dissected into 12-16 pieces, of which 3-4 pieces were used for RNA isolation. The remaining left ventricular pieces were returned to RNA*later*® solution and stored at -80°C. The pieces of left ventricular tissue were syringe homogenised in 1 mL TRIzol© reagent (Life Technologies, Carlsbad, CA, USA) until it passed through an 18- then 21-gauge needle, total RNA was then isolated according to manufacturer's guidelines. Total RNA was further purified using RNeasy spin columns (Qiagen, Limburg, Netherlands). The RNA yield and purity were determined using a NanoDrop ND-1000 (Thermo Fisher Scientific) and diluted to a final concentration of 50 ng/µL. RNA integrity was assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), with RNA integrity (RIN) scores ≥8.0 for each sample (Figure 2.1). Total RNA was stored at -80°C prior to cDNA synthesis. Total RNA was isolated from HL-1 and 3T3 cells in a similar manner using 1 X 10^6 cells per isolation. For cDNA synthesis 500 ng of total RNA was reverse transcribed using the Superscript III First Strand cDNA Synthesis System (Life Technologies) according to manufacturer's protocols. Synthesised cDNA was diluted with nuclease free water 1:20 and stored at -20°C before use.

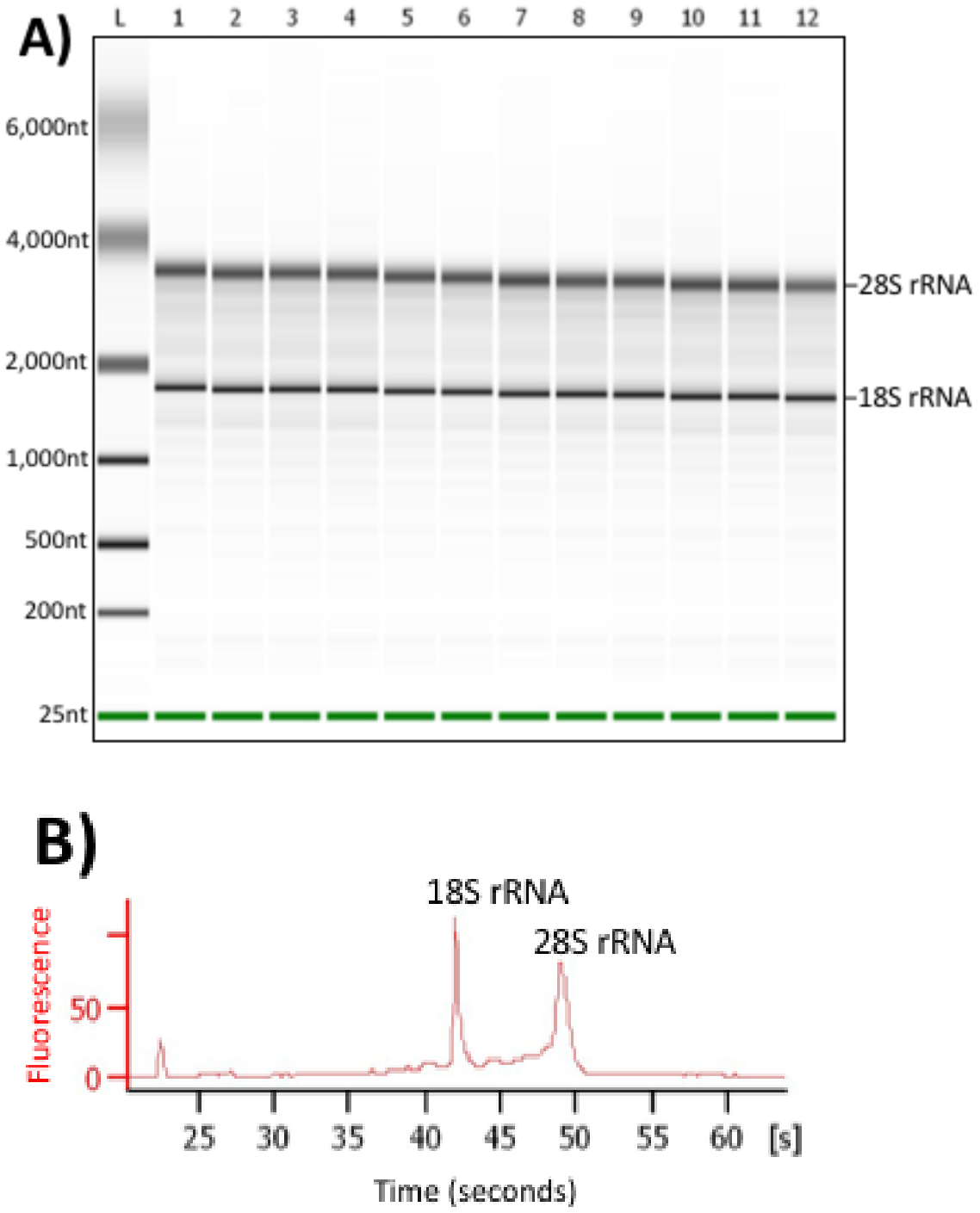


Figure 2.1 RNA integrity of total RNA isolated from murine ventricular tissue A) Representative digital gel image demonstrating 28S and 18S rRNA bands from total RNA isolated from 12 different hearts (Lanes 1-12); RNA size ladder (L). **B)** Representative electropherogram demonstrating good quality RNA showing distinct 28S and 18S rRNA peaks (RIN=8.7).

2.3 Reverse Transcription-quantitative PCR

2.3.1 RT-qPCR

All RT-qPCR studies were conducted using "The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE)" guidelines as outlined by Bustin and colleges (2009). By following MIQE guidelines more reliable and reproducible qPCR results are obtained. Two-step RT-qPCR was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) utilising SYBR Green I chemistry. PCR primers (Table 2.1) were designed with PerlPrimer software (Marshall 2004) according to standard guidelines (Ashton & Headrick 2007). Briefly, primer pairs for each transcript were designed to have a target annealing temperature of 61°C, span an intron/exon boundary and generate an amplicon between 75-150bp in size. The final reaction volume (10 µL) included 1X iQ SYBR Green Supermix (Bio-Rad), 100nM of each primer and 5 µL of diluted (1:20) cDNA. Optimal qPCR cycling conditions consisted of an initial denaturation at 95°C for 3 minutes followed by 40 cycles of 95°C for 15 seconds and 61°C for 60 seconds. After the final PCR cycle, reactions underwent melt curve analysis to detect non-specific amplicons. All reactions were performed in triplicate with each plate containing an equal number of samples from each group, a calibrator control derived from a pool of all cDNA samples, and a no template control (NTC). PCR amplification efficiencies (90-110%) for each primer pair were calculated using a 4-log serial dilution of the calibrator sample. PCR data were analysed using CFX Manager v2.0 (Bio-Rad, Hercules, CA). Following automatic baseline correction, the threshold level was set during the geometric phase of PCR amplification for quantitative measurement (Figure 2.2). The calibrator sample was used to normalise inter-assay variations, with the threshold coefficient of variance for intra- and inter-assay replicates <1% and <5%, respectively. Normalised expression ($\Delta\Delta$Cq) was calculated, with expression normalised to reference gene levels (as determined in Section 2.3.2) and the calibrator control then $\log_2$ transformed.

Table 2.1 RT-qPCR primer sequences.

Gene name	Gene symbol	NCBI GeneID	Forward primer (5'-3')	Reverse primer (5'-3')
Reference Genes				
Actin, beta	*Actb*	11461	ATGAGCTGCCTGACGGCCAGGTCATC	TGGTACCACCAGACAGCACTGTGTTG
Glyceraldehyde-3-phosphate dehydrogenase	*Gapdh*	14433	TGACGTGCCGCCTGGAGAAA	AGTGTAGCCCAAGATGCCCTTCAG
Phosphoglycerate kinase 1	*Pgk1*	18655	AACAACATGGAGATTGGCACATC	AGGCAAGGTAATCTTCACACCATTT
Peptidylprolyl isomerase A	*Ppia*	268373	CGCGTCTCCTTCGAGCTGTTTG	TGTAAAGTCACCACCCTGGCACAT
Ribosomal protein L13A	*Rpl13a*	22121	ATCTTGAGGTTACGGAAACAG	TGAGGCAAACAGTCTTTATTGG
Sex-validation				
Ubiquitously transcribed tetratricopeptide repeat gene, Y chromosome	*Uty*	22290	CGAAATTCAGTTTCATATTGCTCACT	TGATGGCAAGCTTTCAATCTGT
Inactive X specific transcript	*Xist*	213742	ATTGTAGCATACGCATACTTTCAAAGTAT	TGTTTGAACTCCCAGACCTCTTC
Caveolae-related				
Caveolin 1	*Cav1*	12389	AGGTGACTGAGAAGCAAGTG	TCAAAGTCAATCTTGACCACG
Caveolin 2	*Cav2*	12390	TTGACTACGCAGATCCTGAG	GAAGCCTAGCTTGAGATGAG
Caveolin 3	*Cav3*	12391	GGATCTGGAAGCTCGGA	AATCTACCTTCACAATGTCCTC
Cavin 1	*Cavin1*	19285	GATCTACCAGGATGAAGTCAAGC	CTCGTCACCCTCCTTCTCAG
Cavin 2	*Cavin2*	20324	GCTCATCTTCCAGGAAGAAAGTG	AGGGACTTGTTCTCATCAGC
Cavin 3	*Cavin3*	109042	TGCTCTTCAAGGAGGAGACTG	CATCCTCTGGCTGATCTGGG
Cavin 4	*Cavin4*	68016	GTAATCTTCCAGGAGGACATTCC	CCGAGGAGAGCTCTATTGGG
Popeye domain containing 1	*Popdc1*	23828	GATGTTGTCTCTAGGATGTACCC	ATATTGATACCCAAGAACACCGAG
Popeye domain containing 2	*Popdc2*	64082	TTTGAGGAGGTTTCAGGATCAG	GAGGACGTCTAGAGGGTAGG
Popeye domain containing 3	*Popdc3*	78977	GCTCTGAAGTGGTTAGTTTGG	ACTGTCACTCTGATTCTTCCTG

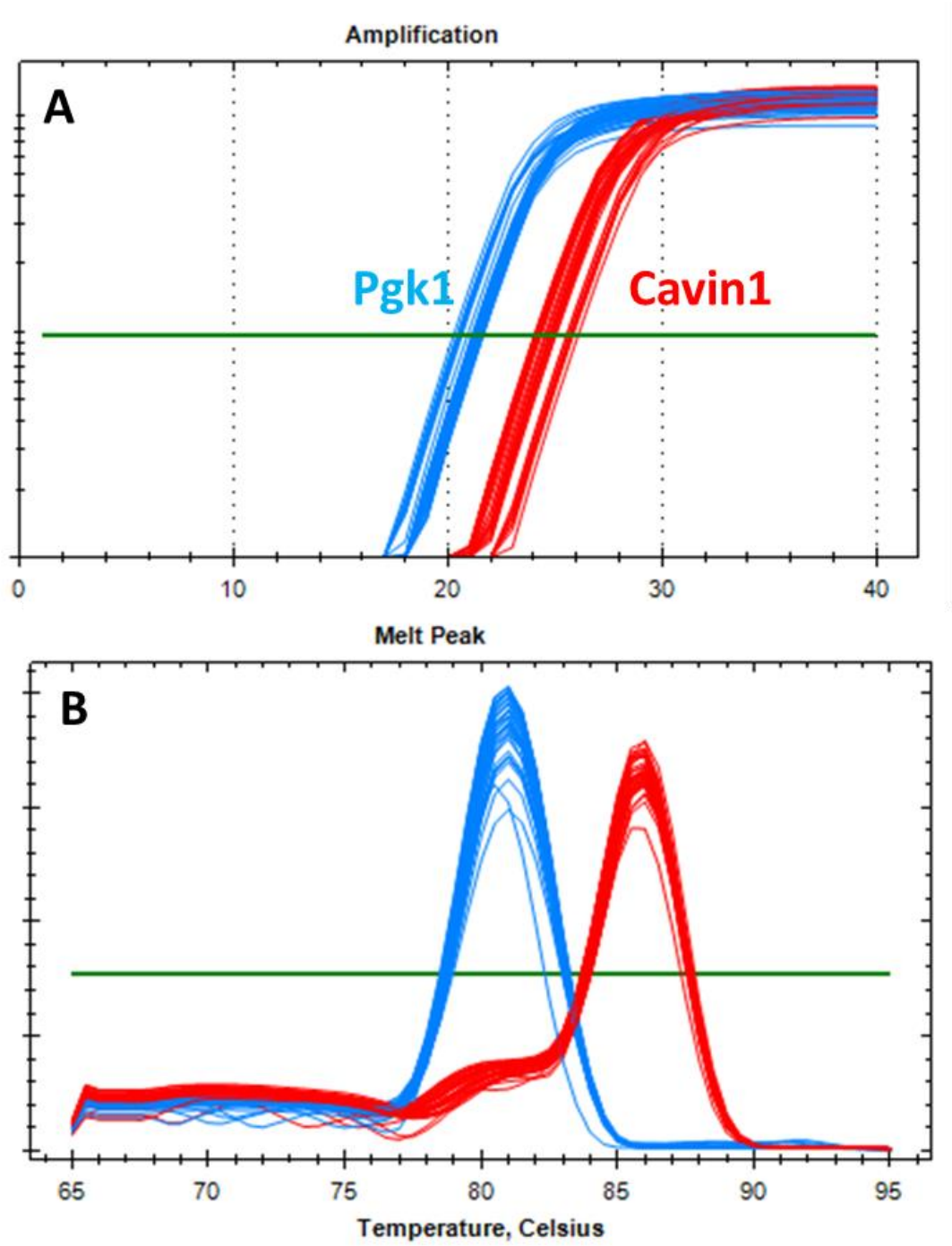


Figure 2.2 RT-qPCR data. A) Amplification plots (semi-log) of *Cavin1* (red) and the *Pgk1* (blue) reference gene. The threshold (green line) is set during the exponential phase of all amplification plots. **B)** Melt-curve analysis of *Cavin1* (red) and the *Pgk1* (blue) reference gene. Data shows single peaks for each curve indicating the presence of a single specific amplicon.

2.3.2 Reference Gene Selection

Due to its practical and conceptual simplicity, qPCR has become the gold standard approach for quantifying gene expression. However, despite raising awareness of potential pitfalls of qPCR, use of experimentally validated reference genes for data normalisation is still widely disregarded (Gutierrez et al. 2008). Using the same general RT-qPCR parameters described in Section 2.3.1, five additional genes (*Actb*, *Gapdh*, *Pgk1*, *Ppi* and *Rpl13a*) were assessed using Bio-Rad CFX Manager Target Stability analysis to determine their usability as reference genes in the ageing male and female hearts. This statistical approach is an iterative test of pairwise variation (Vandesompele et al. 2002). Two putative reference genes were analysed per plate and biological replicates were split into two plates with the results merged using a common calibrator sample. The most stably expressed reference gene displays a stability value (M value) <1.0 for homogenous samples (Vandesompele et al. 2002). *Pgk1* was chosen as the reference gene for all experimental groups as it was found to be stably expressed (M <1.0) across all age groups (8, 16, 32 and 48-week), treatments (normoxic and I-R) and sex (male and female).

2.4 Reference Gene Stability Across Treatment Groups and Sex

To assess the stability of the reference gene between treatment groups (normoxic versus I-R) and sex (male versus female) *Pgk1* transcript expression was further analysed in the baseline 8-week old hearts (n=6/group). Briefly, equal quantities of total RNA (500ng) was used to synthesize cDNA and following RT-qPCR, *Pgk1* expression was normalised to the geometric mean of two (*Rpl13a* and *Ppia*) reference genes. These comparisons were replicated in three separate experiments to demonstrate stability and reproducibility. As shown in Figure 2.3, differences in *Pgk1* reference gene expression was negligible (1.08-fold reduction) in male normoxic and I-R 8-week old baseline hearts. In addition, *Pgk1* expression was stable (1.05-fold increase) in female normoxic and I-R baseline hearts. However, the minor differences in reference gene expression were normalised for to ensure small additive changes did not contribute to the between treatment group analysis. However, comparisons between the sexes showed a dramatic difference in reference gene expression, with female hearts demonstrating a 3.26-fold reduction in *Pgk1* expression levels relative to male matched

hearts. This large variation in reference gene expression therefore prohibits direct statistical comparisons between the sexes, but does enable the direct comparison (following normalisation) of the normoxic and I-R groups within the same sex.

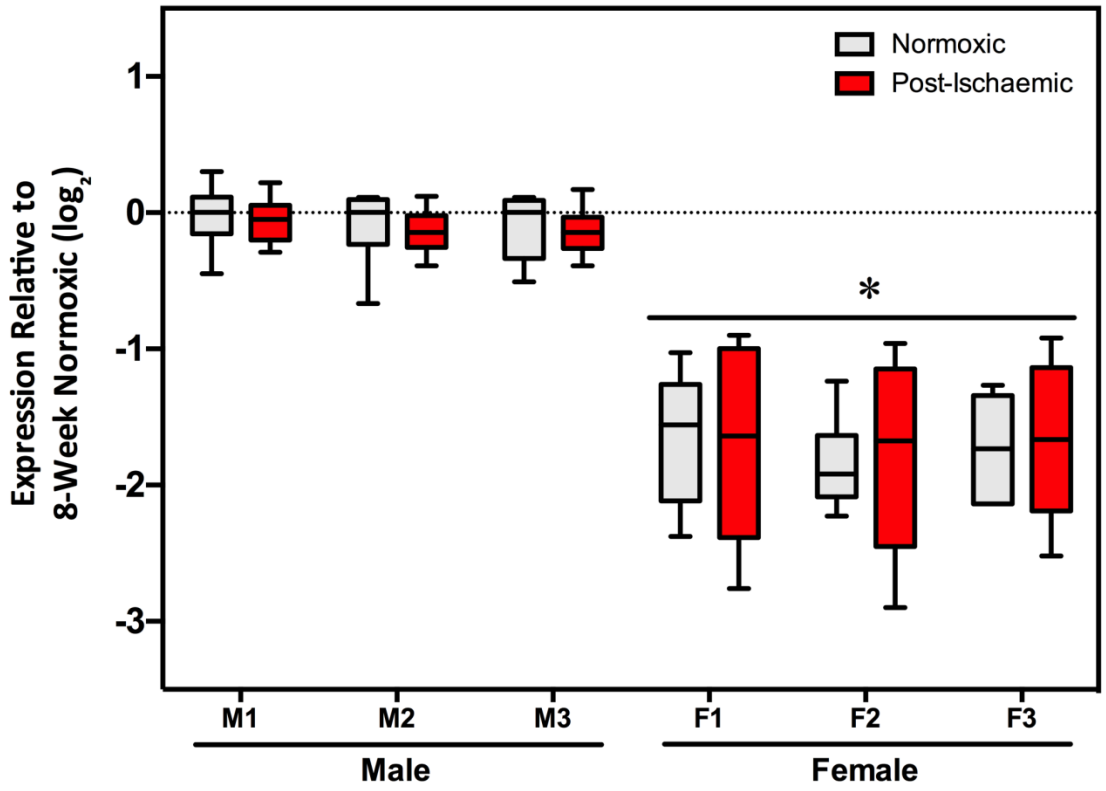


Figure 2.3 Reference gene stability assessment relative to post-ischaemia and/or sex differences in the murine heart. Shown are boxplots detailing gene expression changes determined by RT-qPCR for the *Pgk1* reference gene in the male and female 8-week old heart following normoxic or post-ischaemic stress (n=6/group; triplicated). Boxes indicate interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values. *$P < 0.05$ vs. male hearts.

2.4.1 Sex Validation

Due to the large number of mice used and their difference stages of development we chose to validate their sex to control for incorrect sex-typing. Each sample was assessed for expression of *Uty* and *Xist*, which are highly expressed in males and females respectively. The *Uty* gene is located on the Y chromosome, without an X chromosome homolog and is thus specific to males. Whilst the *Xist* RNA gene is located on the X chromosome and is a major effector of the X inactivation process specific to females. RT-qPCR was carried out according to Section 2.3.1 using *Pgk1* as the reference gene. The ΔCq values (e.g. *Uty* Cq – *Pgk1* Cq) of each gene were plotted on a scatterplot showing that each sample clusters either as male (*Uty* expression) or female (*Xist* expression) as shown in Figure 2.4.

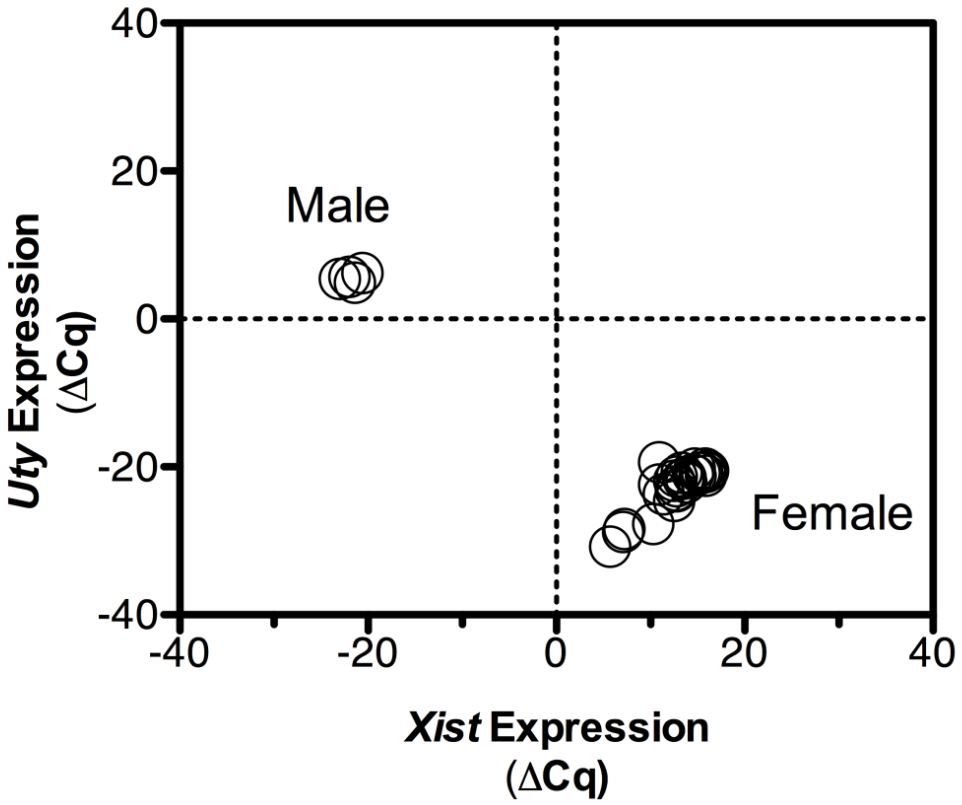


Figure 2.4 RT-qPCR sex typing of mice using *Uty* and *Xist* transcript expression. Each heart clustered specifically to one of two groups confirming the sex of the mice.

2.5 Western Immunoblotting

Immunoblot analysis was employed to confirm myocardial expression of transcripts implicated to change in expression in ageing normoxic and ischaemic hearts. Antibodies for Gapdh, β-actin, Cav3 and Cavin1 were investigated. β-actin (rabbit polyclonal) was obtained from Cell Signalling (Danvers, MA, USA), Cav3 (mouse monoclonal) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA), and Gapdh (rabbit polyclonal) and Cavin1 (rabbit polyclonal) were obtained from Abcam (Cambridge, MA, USA). Briefly, left ventricle tissue (n=6/group) were homogenised using an Ultra Turrax T25a rotor-homogeniser (IKA, Cologne, Germany) in RIPA buffer (Sigma-Aldrich, St Louis, MO) containing protease inhibitors (Sigma-Aldrich, St Louis, MO). Protein content for whole-cell extracts was determined using a Pierce BCA kit (Thermo Fisher, Waltham, MA). Firstly, Gapdh and β-actin protein expression in normoxic and ischaemic aging hearts were analysed for expression stability. β-actin was shown to be more stable in expression relative to Gapdh in normoxic and post-ischaemic groups for both sexes and was chosen as the loading control. Samples (=6/group, singlicate) were prepared in Laemmli buffer containing 30 µg of protein from whole-cell fractions and loaded onto 12% acrylamide gels with equal loading confirmed by Ponceau staining. Electrophoresis was carried out at 75 V for 90 minutes. Protein was transferred to an Immobilon®-FL PVDF membrane (Merck Millipore, Billerica, MA, USA) and blocked in Licor blocking buffer for 120 minutes (Licor, Lincoln, NE, USA). Membranes were then incubated with primary antibody (Cav3, 1:1,500; Cavin1, 1:2000, β-Actin: 1:1,500; Gapdh, 1:2000) overnight at 4°C. Dual coloured labelling was performed using fluorescently labelled secondary antibodies. Briefly, membranes were incubated in a 1:20,000 dilution of IRDye® 680RD donkey anti-rabbit IgG and IRDye® 800CW goat anti-mouse IgG (Licor) secondary antibodies for 120 minutes with gentle agitation. The labelled membranes were washed with TBS and TBST and scanned using an ODYSSEY® CLx infrared scanner (Licor). Densitometric analysis was performed using Image Studio™ 5.0 (Licor) with the protein of interest (Cavin1 or Cav3) normalised to the stably expressed loading control (Figure 2.5). Data were then $\log_2$ transformed relative to 8-week old heart expression.

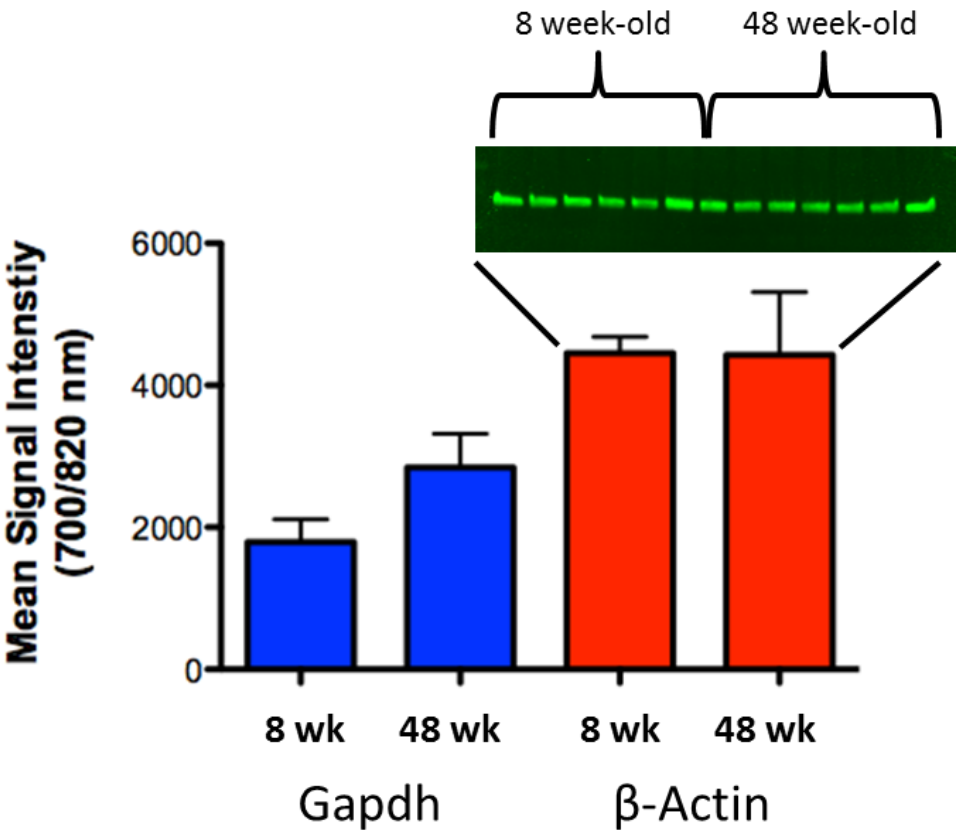


Figure 2.5 Representative image of reference protein expression stability in ageing male post-ischaemic hearts. Gapdh showed induction with ageing although this was not statistically significant (P= 0.11). β-actin was found to be stably expressed in normoxic and post-ischaemic hearts in males and females with representative β-actin image shown in normoxic young (8-week) and middle aged (48-week old) male hearts (n= 6-7/ group).

2.6 Statistical Analyses

Statistical analysis was performed using Prism 6.0 software (GraphPad, California, USA). Data was first assessed for equal variance using Bartlett's test for equal variances. Parametric data comparisons between more than two groups were made via an analysis of variance (ANOVA), with Tukey's post-hoc test applied where differences were detected for gene expression analysis. For data that was determined to be non-parametric a Kruskal-Wallis test was applied. For pair-wise comparisons a two-tailed Student's t-test was employed for Western blotting. For comparisons between age-matched normoxic *vs.* post-ischaemic gene expression data and age-matched male *vs.* female recovery and LDH release, a two-way ANOVA was employed using Prism 6.0 (Graphpad). For comparisons within normoxic and I-R hearts, Tukey's post-hoc test was applied where differences were detected for gene expression analysis. For age-matched comparisons, Sidak's post-hoc test applied where differences were detected for gene expression analysis. Non-parametric data was analysed for significance using a Mann-Whitney test. Statistical significance was accepted for $P < 0.05$.

3. Chapter Three

Effects of age on caveolae-related transcript expression in normoxic and post-ischaemic male hearts

ADDENDUM

Contributions to Chapter 3:

Can Kiessling:

- Experimental design
- All gene expression & protein expression analysis
- Data analysis
- Author of the chapter

Kevin Ashton:

- Assistance with experimental design and interpretation of results
- Editing of chapter

Melissa Reichelt:

- Heart perfusions

Jason Peart:

- Editing of chapter

Selected data from this chapter have been previously published

Kiessling C.J., Reichelt M.E., Headrick J.P., Ashton K.J. (2013). Transcriptional Analysis of Caveolin and Cavin Transcripts in Normoxic and Post-Ischaemic Aged Hearts. Heart, Lung and Circulation. 22:234.

Kiessling C.J., Reichelt M.E., Peart, J., Headrick J.P., Ashton K.J. (2014). Transcriptional Analysis of Caveolin and Cavin in the Male and Female Ageing Mouse Heart Following Ischaemic Stress. Circulation Research. 115: A220.

3.1 INTRODUCTION

Ageing is associated with complex changes at both physiological and molecular levels. The ageing heart in both humans and animal models has been shown to display reduced tolerance to I-R (Willems et al. 2005; Boengler, Schulz & Heusch 2009). This is evidenced by a loss of functional recovery and enhanced cell death, as shown by an increase in lactate dehydrogenase (LDH) efflux (Willems et al. 2005; Peart et al. 2014) or markers such as tetrazolium chloride staining (Schulman, Latchman & Yellon 2001). Furthermore, approaches such as IPC and pharmacological preconditioning which protect the young heart from I-R injury appear ineffective in the aged myocardium (Peart et al. 2014; Boengler, Schulz & Heusch 2009). The molecular mechanisms behind the loss of cardioprotection in the ageing myocardium may include changes in sensitivity of GPCR-mediated signalling, changes in control of mitochondrial permeability, altered autophagy, and other mechanisms as discussed in Section 1.6.5.

Recently, specialised micro-domains within the plasma membrane termed caveolae have been shown to compartmentalise and concentrate various classes of signalling molecules, including important mediators of cardioprotection such as receptor tyrosine kinases, GPCRs and their subunits (Insel et al. 2005). It is hypothesised that ischaemic stress and cardioprotective interventions generate autacoids (e.g. adenosine, opioids, bradykinin) which recruit a number of signalling pathways through activation of their respective receptors and subunits, which are co-localised and enriched in caveolae (Hausenloy & Yellon 2010). Furthermore, cardioprotective stimuli such as IPC have been shown to increase caveolae formation (Tsutsumi et al. 2008). Conversely pharmacological disruption of caveolae impairs ischaemic tolerance in cultured cardiomyocytes, similar to results from sI-R in primary cardiomyocytes, whilst Cav3-deficienct hearts exhibit impaired ischaemic tolerance (See Hoe et al. 2014; Horikawa et al. 2008). The chief structural proteins of caveolae are the caveolins, a family consisting of three members: Caveolin-1, Caveolin-2 and Caveolin-3, encoded by *CAV1*, *CAV2* and *CAV3* genes, respectively (Williams & Lisanti 2004). These proteins form oligomers and associate with cholesterol and sphingolipids in the plasma membrane, leading to the formation of functional caveolae (Scherer et al. 1997). Whilst Cav1 and Cav2 are ubiquitously co-expressed, Cav3 is muscle-specific and responsible for caveolae formation in cardiomyocytes, as confirmed in double Cav1 and Cav3-deficient models (Park et al. 2002).

In terms of ischaemic tolerance, both Cav1 and Cav3 appear to contribute to cardioprotective responses. More specifically, Cav1 peptide has been shown to be cardioprotective while Cav1-deficiency results in attenuated cardioprotection (Young, Ikeda & Lefer 2001; Jasmin et al. 2011). Cardiac-specific overexpression of Cav3 results in enhanced ischaemic tolerance characterised by enhanced recovery and reduced cell death; conversely Cav3-deficiency results in ablation of this protective phenotype (Tsutsumi et al. 2011). The role of Cav2 remains uncharacterised in the heart, although it has been shown to act as an accessory protein by making caveolae more uniform, structurally deeper and more abundant (Fujimoto et al. 2000).

More recently other protein families including the cavins (Cavin1,-2,-3 and -4) and the Popeye domain-containing (Popdc1, Popdc2, Popdc3) proteins have been shown to be localised in caveolae and contribute to caveolae formation and morphology in a tissue-dependant manner (Bastiani et al. 2009; Alcalay et al. 2013). With regards to these two gene families, thus far only Cavin1 and Popdc1 have been shown to influence caveolae formation, with deficiency in Cavin1 and Popdc1 resulting in reductions of caveolae in the heart (Kasahara et al. 2014; Alcalay et al. 2013). At present little is known regarding their expression and functional roles in the heart.

In terms of cardioprotection, Cavin1 has been shown to anchor Trim72, a protein critical for membrane repair following I-R (Zhu et al. 2011; Wang et al. 2010). Knockdown of Cavin2- has been shown to result in hyper activation of the Akt signalling pathway via Phosphatase and tensin homolog attenuation at the sarcolemma (Murayama et al. 2014). In addition, Cavin2 may target Cavin1 recruitment to the plasma membrane and help stabilise caveolae invaginations (Hansen et al. 2009). Cavin3 and Cavin4 are not crucial for caveolae formation in the heart (Hansen et al. 2013; Ogata et al. 2014). Cavin4 has been shown to be up-regulated in response to hypoxia, and is associated with hypoxia-induced hypertrophy in cardiomyocytes (Shyu et al. 2014). Cavin4 expression is perturbed in rippling muscle diseases concomitant with loss of Cav3 (Bastiani et al. 2009).

With regards to the Popdc family, Popdc1-deficient mice demonstrate a loss of pharmacological preconditioning and impaired recovery following I-R, perhaps due to significantly reduced caveolae formation in these hearts (Alcalay et al. 2013), whilst a role for

Popdc2 remains uncharacterised in the heart. Very little is known about the role of Popdc2 in the heart except that it is essential for normal heart formation and is enriched in the cardiac pacemaker system, whilst the function of Popdc3 remains un-investigated (Kirchmaier et al. 2012; Schindler et al. 2012). It is likely that Popdcs have critical roles in the heart as differential expression of Popdcs has been observed in the context of heart failure (Gingold-Belfer et al. 2011).

Surprisingly little is presently known about these caveolae-related proteins in the ageing myocardium. However, Cav3 and caveolae appear to be reduced in the aged heart in association with a reduced efficacy of pharmacological and ischaeamic preconditioning (Peart et al. 2014). The expression pattern of other caveolae-associated proteins remains uncharacterised in the ageing heart. Thus, we sought to investigate the expression of caveolin, cavins and the Popdc families in the ageing normoxic and post-ischaemic male heart.

3.2 METHODS

Langendorff perfusions were carried out on hearts (n=8-10/per group) from 8 (young-adult), 16 (mature-adult), 32 (adult) and 48 weeks old (middle-aged) male mice as previously described in Section 2.1.1. Briefly, 20 min of global normothermic ischaemia was followed by 60 min of reperfusion whilst normoxic hearts were maintained under aerobic perfusion for 80 min.

RNA isolation and cDNA synthesis from normoxic and post-ischaemic left ventricular tissue was carried out as described in Section 2.2.1. RT-qPCR was used to analyse expression of caveolin, cavin and Popdc transcripts in ageing hearts as detailed in Section 2.3.1. Selection of a stable reference gene was carried out according to Section 2.3.2. Normalisation of post-ischaemic gene expression to normoxia was carried out according to Section 2.4.

Western immunoblotting was carried to confirm validate the relationship between transcript and protein expression changes for Cav3 and Cavin1 according to Section 2.5.

Statistical analysis was conducted as detailed in Section 2.6, with statistical significance accepted for $P<0.05$.

3.3 RESULTS AND DISCUSSION

3.3.1 Analysis of mRNA abundance in left ventricular tissue and 3T3 and HL-1 cell lines

The heart is a heterogeneous tissue containing numerous cell types including cardiomyocytes, fibroblasts, vascular smooth muscle and endothelium, and varied immune cells. Our initial goal was to elucidate the relative abundance of transcripts in left ventricular cardiac tissue (from normoxic 8-week old male hearts) and compare these profiles to those for both cultured HL-1 cardiomyocytes and 3T3 fibroblasts. Expression values were normalised to *Pgk1* as a reference gene, which was expressed at similar levels (<0.5 Cq difference) within left ventricular tissue, HL-1 cardiomyocytes and 3T3 fibroblasts.

As shown in Figure 3.1 all caveolin transcripts are expressed in cardiac tissue. *Cav1* was found in similar abundance in 3T3 and HL-1 cells. Interestingly, *Cav2* expression was low in both HL-1 and 3T3 cells when compared to left ventricular tissue. *Cav3* expression was modest in HL-1 cardiomyocytes and was not detected in 3T3 fibroblasts, confirming the muscle-specific expression of *Cav3*.

Similar to caveolins, all cavin transcripts were modestly expressed in the heart relative to *Pgk1* (>40%). *Cavin3* was the most abundant cavin in the heart. Relative expression in this study resembles the protein expression of caveolin and cavin for whole heart lysates in prior studies (Bastiani *et al*. 2009; Hansen *et al*. 2013). Similar to the heart, HL-1 cardiomyocytes and 3T3 cells showed comparable *Cavin1* and *Cavin2* expression. *Cavin3* cardiac expression appears to be predominantly from cardiomyocytes rather than fibroblasts. Levels of *Cavin4* expression in HL-1 cardiomyocytes and 3T3 fibroblasts were notably less than in left ventricular tissue. This difference may reflect major contributions from cells other than cardiomyocytes and fibroblasts in the whole myocardial signal. Alternatively, the difference may reflect changes in expression due to cell line immortalisation, or their original tissue source.

Cardiac tissue and HL-1 cardiomyocytes showed moderate *Popdc1* expression, with expression of *Popdc2* the most abundant (90% relative to *Pgk1*) transcript of those investigated. This is consistent with high expression of Popdc2 reported in other studies (Andree *et al*. 2000). Cultured fibroblasts did not express *Popdc1* and *Popdc2*, further confirming the muscle-specific expression of *Popdc* transcripts. Although *Popdc3* was expressed in the heart, there was no detectable *Popdc3* expression in cultured

cardiomyocytes or fibroblasts. It is possible that *Popdc3* is selectively expressed in ventricular but not in atrial cardiomyocytes (e.g. HL-1 cells), with such differential expression previously reported by others (Barth et al. 2005). In addition, *Popdc3* could potentially be missing in the HL-1 cardiomyocytes which has been documented for beta-tubulin II - present in adult primary atrial but not in HL-1 cells (Kuznetsov et al. 2014).

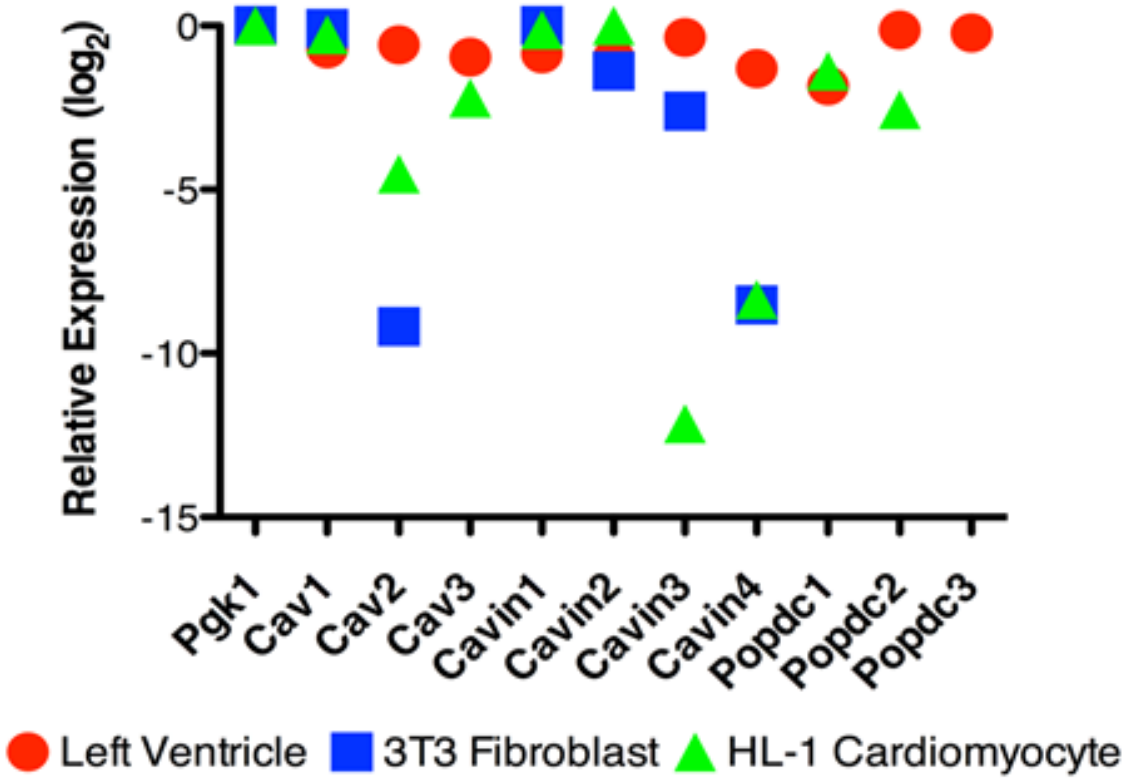


Figure 3.1 Relative expression of Caveolin, Cavin and Popdc transcripts in left ventricle, HL-1 cardiomyocytes and 3T3 fibroblasts. The dot-plot presents relative Caveolin, Cavin and Popdc gene expression levels in left ventricular tissue (8-week old male mice), cultured HL-1 cardiomyocytes and cultured 3T3 fibroblasts. Data presented as means relative to *Pgk1* expression. (n=3/group).

3.3.2 Age dependence of myocardial ischaemia-reperfusion injury in males

The average life-span amounted males are 125 weeks for males and for females 113 weeks with no animals normally living beyond past 140 weeks of age (Kunstyr & Leuenberger 1975). The time points of adult and aged mice can vary between studies as it is difficult to define what characteristics should constitute this. Generally, the 8-week and 16-week old mice have been defined as the young and young-mature (respectively) as they reach sexual maturity at this time point. The 32-week old is defined as the mature adult which exhibits some age-related changes, whilst 48-week old is defined as the middle-aged as it approaches reproductive senescence which occurs past 52-week old. In terms of human years, the 8-week old mice represent ≤20 years old, 16-week with ≈20-years old, 32-week with 27-years old and 48-week with 38-years old as suggested by Jackson Laboratory for C57/Bl6 mice (Jackson Laboratory 2011).

Functional resistance to ischaemia-reperfusion was assessed by measuring recoveries of left ventricular developed pressure in perfused hearts. As shown in Figure 3.2A an age-related reduction in functional recovery became apparent in 32 and 48-week old hearts. Additionally, post-ischaemic LDH release was enhanced in 48-week old hearts, indicating an increase in cell damage and death (Figure 3.2B).

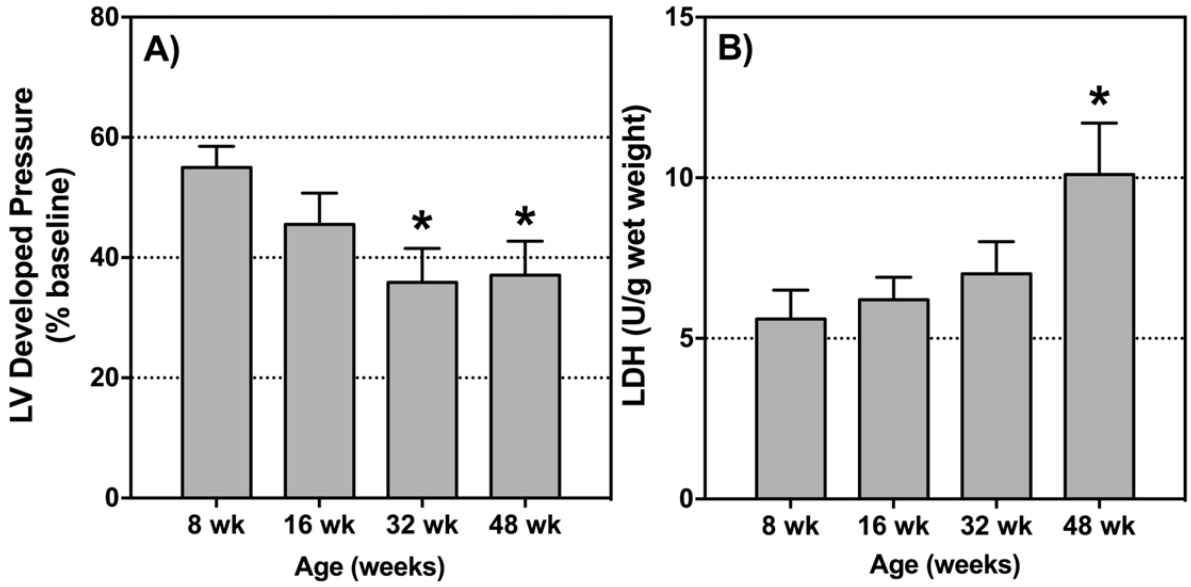


Figure 3.2 Myocardial I-R injury in the ageing male heart. Data are shown for: **A)** left ventricular developed pressure; **B)** cell death following 20 min ischaemia and 60 min reperfusion in isolated hearts from 8, 16, 32 and 48-week old male mice Data are expressed as mean±SEM; **P* < 0.05 vs. 8-week old group. (n=8-12/group).

3.3.3 Reference Gene Stability Analysis in Normoxic and Post-Ischaemic Hearts

As mentioned in section 2.3.2, use of a stably expressed gene is crucial for accurate data normalisation in reverse transcription polymerase chain reaction (RT-qPCR). Five commonly used genes were assessed for stability in normoxic and post-ischaemic hearts (8, 16, 32 and 48-week old). Specifically, β-actin (*Actb*), Glyceraldehyde 3-phosphate dehydrogenase, (*Gapdh*), Phosphoglycerate Kinase 1 *(Pgk1)*, Cyclophilin peptidylprolyl isomerase *(Ppia),* and Ribosomal Protein L13a *(Rpl13a).* Stability analysis showed that all reference genes assessed were stably expressed (M value <1.0) in both normoxic (Figure 3.3A) and post-ischaemic tissue (Figure 3.3B), with *Pgk1* shown to exhibit the most stable expression.

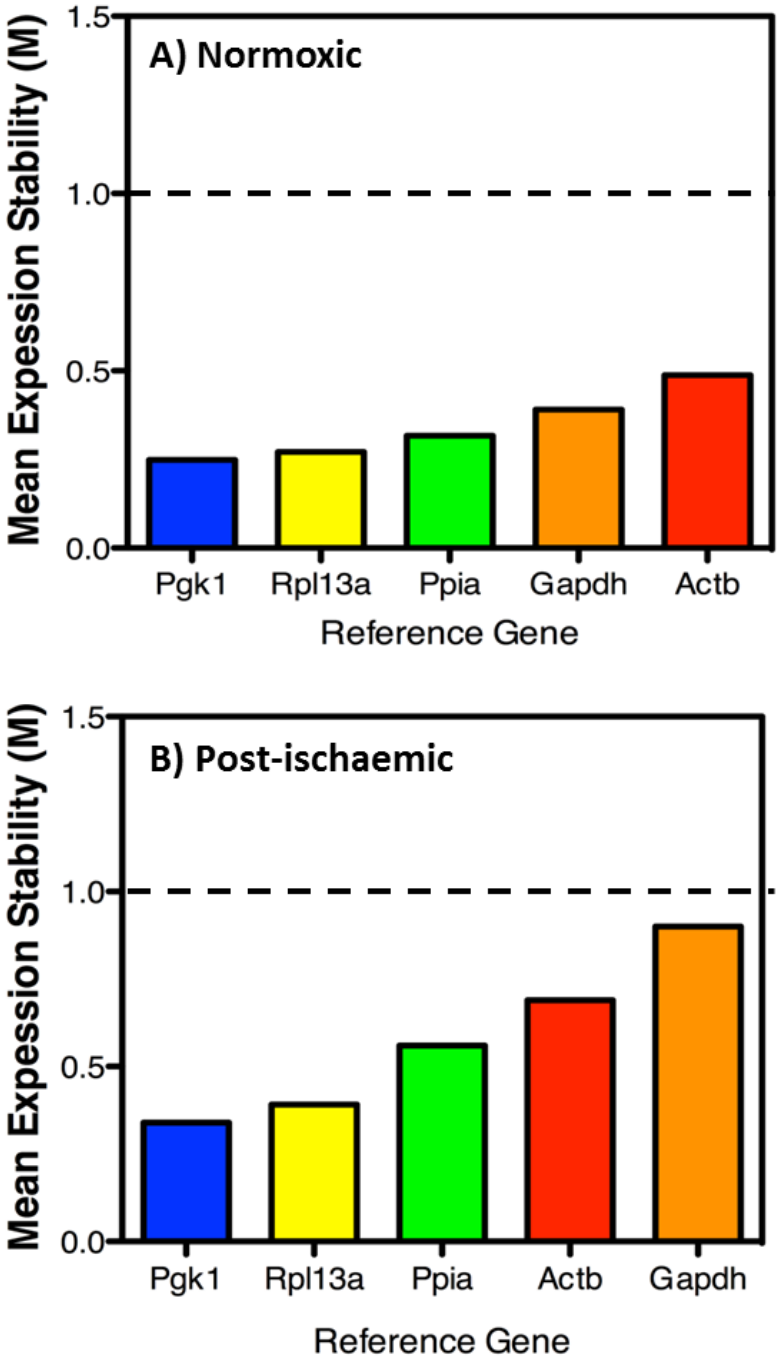


Figure 3.3 Expression stability values (M) of the putative reference genes tested in the male **A)** normoxic ageing hearts and **B)** post-ischaemic ageing hearts. The dotted line indicates the recommended threshold value of <1.0 for heterogeneous samples. (n=24/group).

3.3.4.1 Transcriptional analysis of caveolin expression

RT-qPCR was used to assess the transcript expression of the three caveolin family members in the ageing heart under normoxic conditions and following I-R. Two-way ANOVA yielded a significant main effect for age and *Cav1* mRNA expression (Table 3.1). As shown in Figure 3.4A the expression of *Cav1* is reduced in 48-week old hearts relative to all other ages, this is most pronounced under normoxic conditions. Specifically, *Cav1* declined 1.8-fold in the 48-week old normoxic heart compared to the 8-week old group (Figure 3.4A). A similar shift in expression for *Cav2* was also observed, with two-way ANOVA also identifying a significant age effect. This effect was also identified as a down-regulation (1.4-fold) of expression in 48-week old normoxic hearts (Figure 3.4B). Analysis of *Cav3* expression yielded a significant main effect for both age and ischaemia. The interaction of age and ischaemia was also significant, indicating that the age effect was greater in the normoxic group when compared to post-ischaemic hearts (Table 3.1). This can be further observed in Figure 3.4C, which shows *Cav3* mRNA was down-regulated 1.5-fold as early as 16-weeks in the normoxic heart, with a plateau in expression through 32- and 48-weeks. However, in the post-ischaemic heart *Cav3* expression is induced above baseline (1.5-fold). As the heart ages this response appears to be lost and an age-related decline in *Cav3* expression is observed in a similar manner to their normoxic counterparts.

Table 3.1 Two-way analysis of variance of age and post-ischaemic effects on caveolin transcript expression

Gene	Factor	Mean Square	F (DFn, DFd)	% of Total Variation	*P* value
Cav1	Age	0.661	5.56 (3, 40)	25.6%	**0.0028**
	Ischaemia	0.068	0.57 (1, 40)	0.9%	0.4547
	Age*Ischaemia	0.309	2.60 (3, 40)	12.0%	0.0658
Cav2	Age	0.392	4.69 (3, 40)	24.7%	**0.0067**
	Ischaemia	0.056	0.67 (1, 40)	1.2%	0.5348
	Age*Ischaemia	0.062	0.74 (3, 40)	3.9%	0.4173
Cav3	Age	0.426	13.33 (3, 40)	27.4%	**< 0.0001**
	Ischaemia	1.050	32.87 (1, 40)	22.5%	**< 0.0001**
	Age*Ischaemia	0.352	11.01 (3, 40)	22.6%	**< 0.0001**

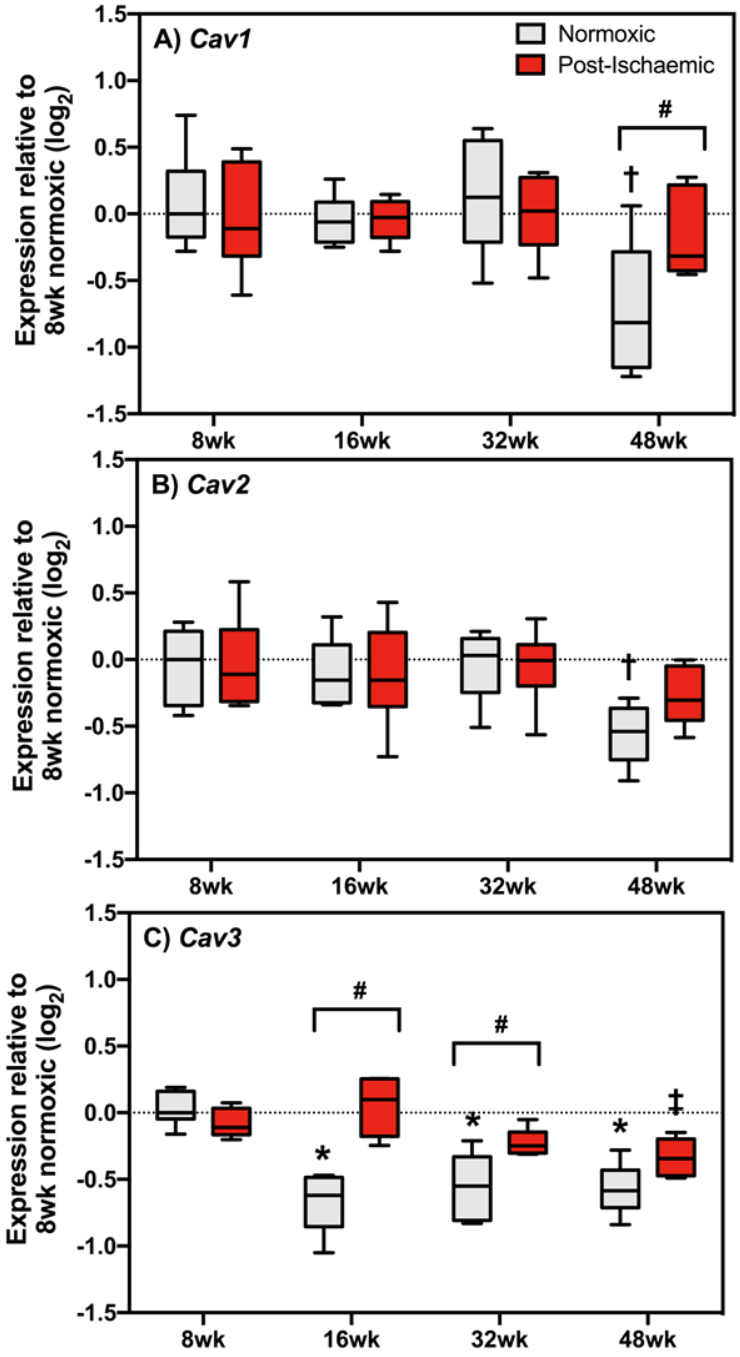


Figure 3.4 Age dependent expression of caveolin transcripts in normoxic and post-ischaemic male hearts. Shown are boxplots detailing expression changes determined by RT-qPCR for **A)** *Cav1,* **B)** *Cav2* and **C)** *Cav3* in the normoxic (grey boxes) and post-ischaemic (red boxes) heart. Boxes indicate interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values. * $P < 0.05$ vs 8-week old normoxic; † $P < 0.05$ vs 8-, 16- and 32-week old normoxic; ‡ $P < 0.05$ vs 16-week old post-ischaemic; # $P < 0.05$ vs age-matched post-ischaemic group (indicated by brackets). (n=6/group).

3.3.4.2 Immunoblot analysis of caveolin3 protein expression

To verify the relationship between *Cav3* transcript and protein expression changes immunoblot analysis was employed. A 1.5-fold reduction in Cav3 protein expression in normoxic hearts was observed in the 48 vs. 8-week old group (Figure 3.5A). Age-dependent Cav3 protein repression (1.8-fold) was also observed in post-ischaemic hearts (Figure 3.5B). This was consistent with the age-related decrease in *Cav3* mRNA expression (Figure 3.4C).

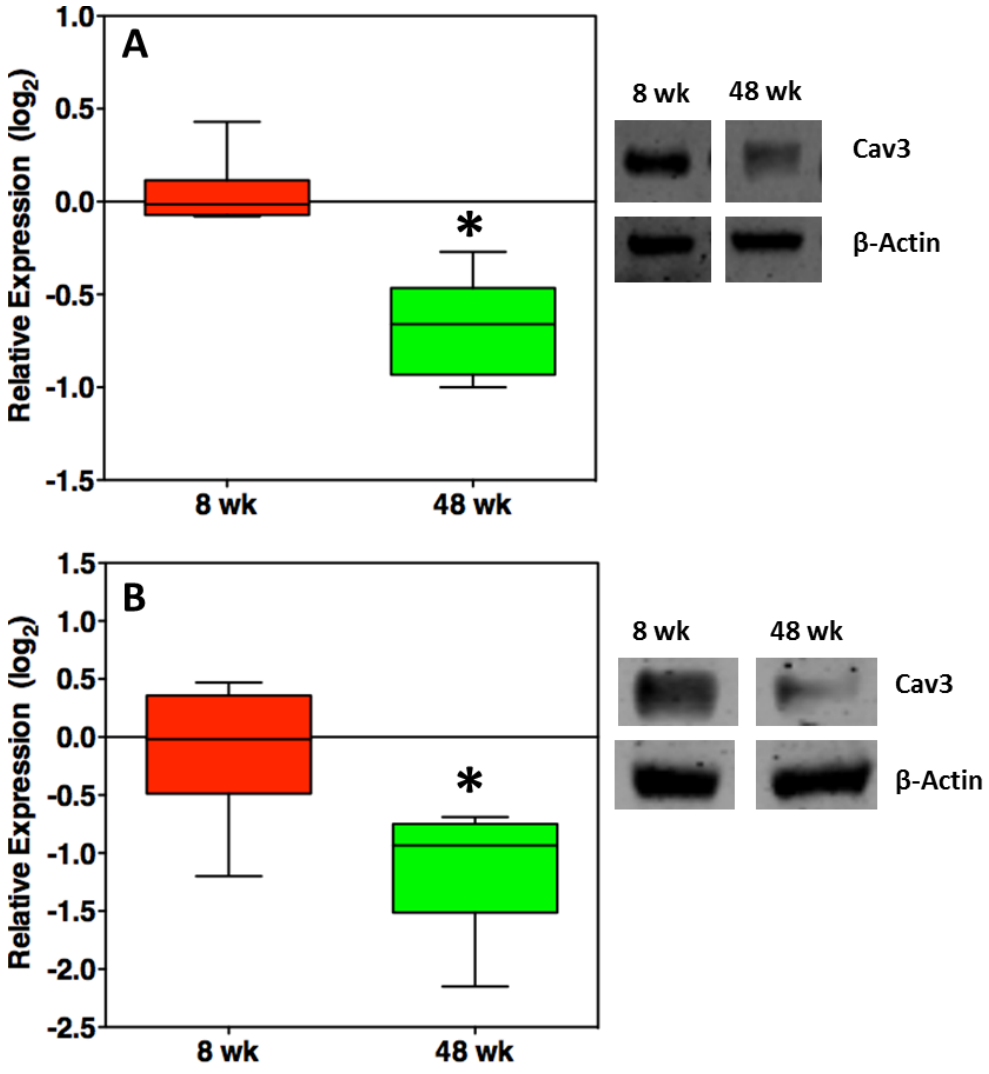


Figure 3.5 Age dependent Cav3 protein expression in normoxic and post-ischaemic male hearts. Boxplots are shown detailing Cav3 protein expression in **A)** normoxic and **B)** post-ischaemic ageing hearts. Boxes indicate the interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values. To the right of the graphs representative immunoblot images are shown for Cav3 and the β-Actin loading control. * $P < 0.05$ vs 8-week old. (n=6/group).

Of the caveolin transcripts, only *Cav3* showed significant down-regulation in both normoxic and post-ischaemic ageing hearts. Although Cav3 has previously been shown to be down-regulated in 74-week old senescent hearts (Peart et al. 2014), here we show down-regulation is apparent as early as 16-week in normoxic hearts, and in the 32 and 48-week post-ischaemic hearts. The age-related decrease in *Cav3* expression correlates with a loss of ischaemic tolerance in 32 and 48-week old post-ischaemic hearts, as shown by LDH release and functional measurements (Figure 3.2A and B). The reduction in Cav3 protein expression in these hearts may lead to reduced caveolae formation, which may contribute to loss of ischaemic tolerance in these hearts when compared to young and mature counterparts. This is a novel finding, as the age-dependent expression of Cav3 in normoxic and post-ischaemic hearts has been uncharacterised until now.

Caveolae formation in the heart is directly dependent on Cav3, with Cav3-deficiency and Cav3-overexpression resulting in ablation and increased caveolae formation, respectively (Park et al. 2002; Horikawa et al. 2011). Loss of caveolae formation via Cav3-deficiency is associated with loss of IPC, anaesthetic and pharmacological preconditioning in these hearts (Tsutsumi et al. 2008; Tsutsumi et al. 2010). The aged heart also exhibits reduced sensitivity to various GPCR receptors, including A_1AR, Bradykinin, and δ_1-opioid receptors, which reduce I-R injury in young hearts but not aged hearts (Headrick et al. 2003; Peart et al. 2007). Concomitant with loss of GPCR-mediated protection is a reduction of Cav3 which we and others (Peart et al. 2014) have observed in older hearts. As many cardioprotective receptors and their signalling subunits are co-localised in caveolae, a reduction in caveolae may play a key role in insensitivity to GPCR stimuli. In support of this view, both Cav3-deficiency and pharmacological disruption of caveolae results in loss of endogenous ischaemic tolerance as well as loss of IPC and pharmacological preconditioning (Tsutsumi et al. 2008; See Hoe et al. 2014). Conversely, cardiac-specific overexpression of Cav3 has been shown to improve recovery of the aged heart, which results in 60–65% recovery of pressure development and end diastolic pressure reportedly in the normal range (Kidd et al. 2010). Furthermore, Cav3-OE inhibits (via ANP) hypertrophy and fibrosis in response to aortic constriction, with enhanced survival rates (compared to wild-types), further supporting involvement of Cav3 and caveolae in stress-resistance in the heart (Horikawa et al. 2011). Taken together, data are consistent with some role for caveolae in age-dependant repression of I-R tolerance and loss of cardioprotection in the aged heart.

3.3.5.1 Analysis of the age-dependence of cavin gene expression in normoxic and post-ischaemic male mouse hearts

Significant down-regulation (1.6-fold) of *Cavin1* was apparent in both the normoxic 16- and 48-week old compared to the 8-week group (Figure 3.6A). In the post-ischaemic heart, a significant reduction (1.3-fold) in *Cavin1* was observed in the 16- and 48-week old groups (Figure 3.6A). Two-way ANOVA analysis of this age-related reduction of *Cavin1* is further demonstrated identifying age as a significant main effect (Table 3.2). *Cavin2* expression in the normoxic heart was significantly up-regulated (1.4-fold) at 32-weeks with expression returning to baseline levels in the 48-week old heart (Figure 3.6B). *Cavin2* also demonstrated a small (1.2-fold) but significant reduction in the 48-week old post-ischaemic heart (Figure 3.6B) and was the only cavin transcript to show significant interaction between age and ischaemia (Table 3.2). More specifically, *Cavin2* showed significant repression in the post-ischaemic 16-, 32- and 48-week old heart when compared to age-matched normoxic counterparts (Figure 3.6B). *Cavin3* showed no age-dependent changes in expression in the normoxic and post-ischaemic heart. While *Cavin4* exhibited a 1.5-fold increase in expression at 48-week relative to 16-week old normoxic hearts, no other significant changes were observed (Figure 3.6D). However, post-hoc analysis of *Cavin4* revealed the reduction of 48-week old post-ischaemic vs 48-week old normoxic hearts was borderline non-significant (P= 0.06)

Table 3.2 Two-way analysis of variance of age and post-ischaemic effects on cavin transcript expression

Gene	Factor	Mean Square	F (DFn, DFd)	% of Total Variation	*P* value
Cavin1	Age	1.014	11.03 (3.40)	43.2%	**< 0.0001**
	Ischaemia	0.192	2.10 (1,40)	2.7%	0.1555
	Age*Ischaemia	0.043	0.46 (3,40)	1.8%	0.7090
Cavin2	Age	1.184	5.25 (3,40)	14.4%	**0.0038**
	Ischaemia	0.018	35.30 (1.40)	32.4%	**< 0.0001**
	Age*Ischaemia	0.202	6.02 (3,40)	16.6%	**0.0017**
Cavin3	Age	0.131	1.38 (3,40)	9.1%	0.2626
	Ischaemia	0.001	0.08 (1,40)	0.2%	0.7740
	Age*Ischaemia	0.050	0.52 (3,40)	3.4%	0.6699
Cavin4	Age	0.120	2.46 (3,38)	12.8%	0.0774
	Ischaemia	0.008	0.16 (1, 38)	0.3%	0.6914
	Age*Ischaemia	0.196	4.01 (3.38)	20.9%	**0.0142**

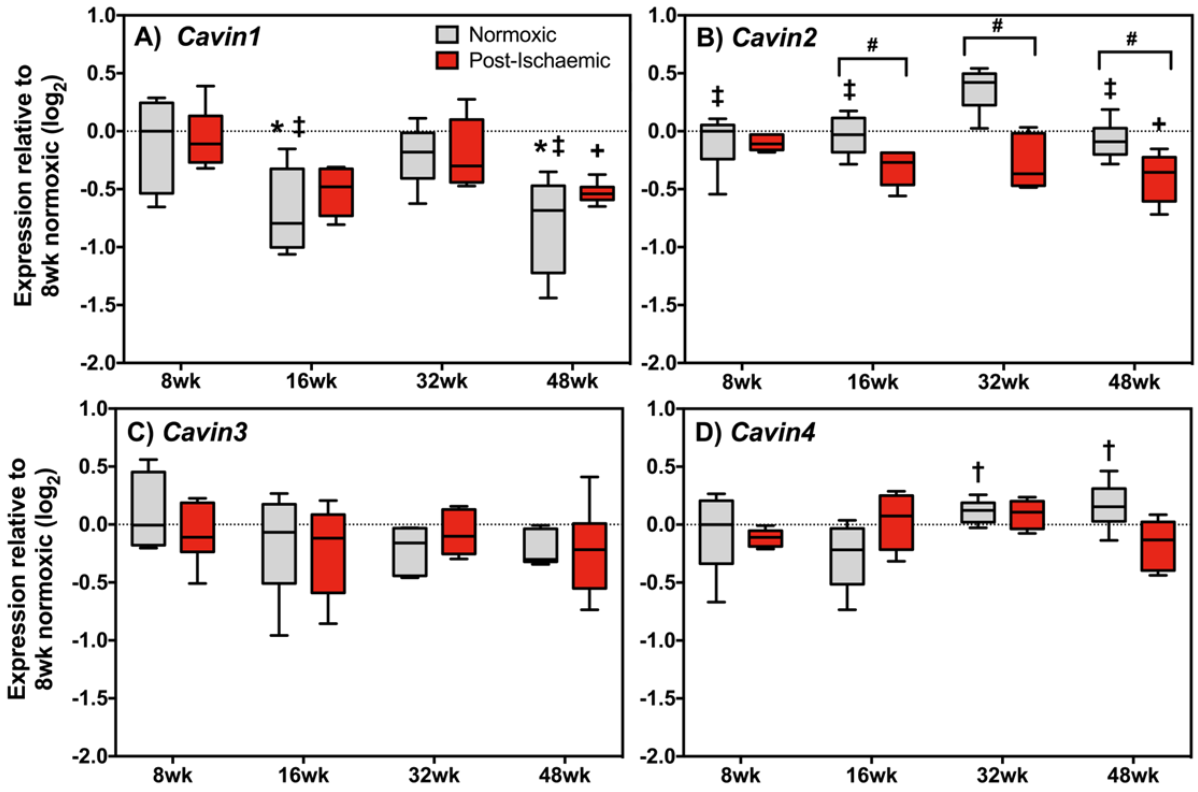


Figure 3.6 Age dependent expression of cavin transcripts in normoxic and post-ischaemic male hearts. Shown are boxplots detailing expression changes determined by RT-qPCR for **A)** *Cavin1,* **B)** *Cavin2,* **C)** *Cavin3* and **D)** *Cavin4* in the normoxic (grey boxes) and post-ischaemic (red boxes) heart. Boxes indicate interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values. * $P < 0.05$ vs 8-week old normoxic; † $P < 0.05$ vs 16-week old normoxic, ‡ P vs 32-week old normoxic, + $P < 0.05$ vs 8-week old post-ischaemic, # $P < 0.05$ vs age-matched post-ischaemic group (indicated by brackets). (n=6/group).

3.3.5.2 Immunoblot analysis of Cavin1 protein expression

To assess whether the age-related reduction in *Cavin1* mRNA expression was translated to the protein level, immunoblot analysis was employed. In the normoxic heart Cavin1 protein was down-regulated (1.4-fold), although borderline non-significant (*P*=0.051) (Figure 3.7A). However, in the post-ischaemic heart an age-related decline (1.6-fold) in Cavin1 protein expression was statistically significant as shown in Figure 3.7B. Collectively these data confirm that *Cavin1* transcript expression changes (Figure 3.6D) translate to shifts in myocardial Cavin1 protein expression.

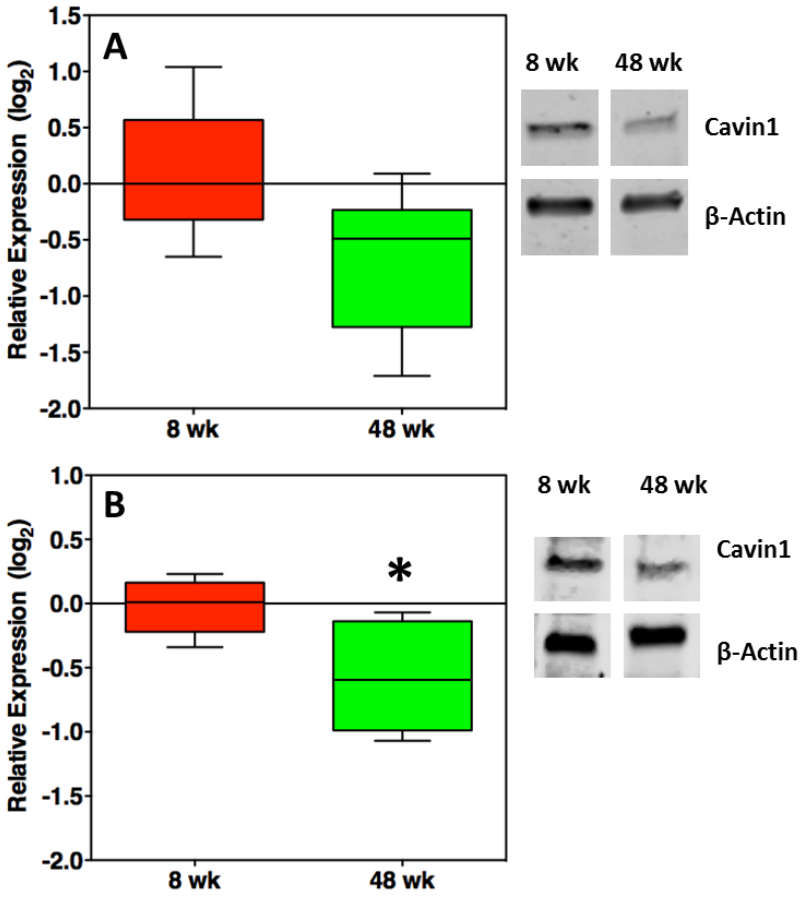


Figure 3.7 Age dependent expression of Cavin1 protein in normoxic and post-ischaemic male hearts. Validation of transcript and protein expression changes for left ventricular Cavin1 in **A)** normoxic and **B)** post-ischaemic ageing hearts. Boxes indicate the interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values. To the right of the graphs representative immunoblot images are shown for Cavin1 and the β-Actin loading control. *$P < 0.05$ *vs.* 8-week old. (n=6/group).

As the expression patterns of cavins have not been characterised in the ageing heart, we sought to assess the age-dependence of cavin expression, which is important in caveolae biogenesis and potentially influences caveolae dependent cardioprotection. Compared to their caveolin counterparts the functional roles of cavins in the heart remain largely uncharacterised. As shown in Figure 3.1, the heart demonstrates high expression of all cavin transcripts, in agreement with proteomic analysis in heart (Bastiani et al. 2009). Of the cavin transcripts, only *Cavin1* and *Cavin2* showed significant age-related down-regulation. *Cavin1* and *Cavin2* exhibit moderate expression levels in left ventricle and HL-1 cardiomyocytes (Figure 3.1), cardiac tissue also demonstrates moderate expression of these at the protein level (Bastiani et al. 2009). Similar to Cav3, Cavin1 appears essential for caveolae formation in muscle cells, with Cavin1-deficiency or mutation leading to the loss of caveolae in cardiomyocytes and skeletal muscle (Kasahara et al. 2014; Hayashi et al. 2009). Disturbed caveolae formation in Cavin1-deficient hearts also impacts Cav3 expression, as these hearts display reduced Cav3 protein expression compared to wild types (Bastiani et al. 2009). In further support, Cavin1-deficient animals show 80%-90% loss of caveolin proteins in all tissues despite upregulated expression of caveolin mRNAs (Liu et al. 2008). Similarly, mutations in CAVİN1 are associated with mis-localisation of all three caveolin family members and reduced caveolae in skeletal muscle of patients with muscular dystrophy (Hayashi et al. 2009).

Besides its role in influencing caveolae formation, recent studies suggest that Cavin1 is also important in targeting plasma membrane repair machinery to sites of injury. Cavin1 co-localises with Trim72, which has been shown to be critical for membrane repair following I-R, leading to cardioprotection (Zhu et al. 2011; Cao et al. 2010). The role of Cavin1 in this repair mechanism suggests that Cavin1 helps transport and bind Trim72 to cholesterol-rich regions (which may be caveolae) thereby anchoring the Trim72 molecule to enable membrane repair (Zhu et al. 2013). This repair response was shown to be reliant on presence of Cavin1 and lipid rafts/caveolae, as cholesterol depletion via MβCD disrupts the repair response (Zhu et al. 2011; Wang et al. 2010). Cavin1 knockdown also leads to a loss of Trim72-mediated responses following membrane damage (streptolysin O), and enhances cellular LDH release and FM1-43 dye entry, further implicating caveolae involvement in mediating the plasma membrane repair response (Zhu et al. 2011). In both normoxic and post-ischaemic hearts studied here, Cavin1 was down-regulated both at the mRNA and protein levels in 48-week old hearts which

also correlated with the loss of ischaemic tolerance in these hearts. Taken together, these data suggest age-related downregulation of Cavin1 in the older hearts (normoxic or post-ischaemic) may lead to reduced Cav3 expression, perhaps through aberrant Cav3 trafficking to the membrane, which may result in loss of morphological caveolae structures. In addition, plasma membrane repair mechanisms may also be impaired in these hearts, which rely on caveolae and its structural proteins (Further discussed in detail later in Chapter 7).

3.3.6 Analysis of the age-dependence of Popdc gene expression in normoxic and post-ischaemic male mouse hearts

In the normoxic heart, cardiac expression of *Popdc1, Popdc2* and *Popdc3* did not change with age (Figure 3.8). In the post-ischaemic heart, *Popdc1* showed a significant decrease 1.4-fold decrease in expression in 32- and 48-weeks of age (Figure 3.8A), similar to expression pattern observed with *Cav3*. *Popdc2* showed no expression changes with age in the post-ischaemic heart (Figure 3.8B). As shown by two-way ANOVA analysis, *Popdc1* expression yielded a significant main effect for age and ischaemia, similar to that of *Cav3* (Table 3.3). This is demonstrated by the significant 1.3-fold down-regulation of *Popdc1* transcript in 32- and 48-week old post-ischaemic hearts when compared to their age-matched normoxic counterparts (Figure 3.8A). Whilst individually not significant, an age-related decline in *Popdc3* expression was observed in the post-ischaemic heart as shown in table 3.3. Two-way ANOVA confirmed a significant main effect for ischaemia and *Popdc3* transcript repression. This repression is reversed in the ageing normoxic heart as reported by the significant interaction between age and ischaemia. More specifically, we observed significant 2.0-fold down-regulation of *Popdc3* in the 32- and 48-week old hearts when compared to their normoxic age-matched counterparts (Figure 3.8C).

Table 3.3 Two-way analysis of variance of age and post-ischaemic effects on *Popdc* transcript expression

Gene	Factor	Mean Square	F (DFn, DFd)	% of Total Variation	*P* value
Popdc1	Age	0.211	4.88 (3,37)	18.5%	**0.0059**
	Ischaemia	0.674	15.56 (1,37)	19.7%	**0.0003**
	Age*Ischaemia	0.157	3.63 (3,37)	13.8%	**0.0216**
Popdc2	Age	0.363	1.21 (3,37)	8.4%	0.3184
	Ischaemia	0.133	0.44 (1,37)	1.0%	0.5091
	Age*Ischaemia	0.215	0.72 (3,37)	5.0%	0.5486
Popdc3	Age	0.028	0.19 (3,35)	0.8%	0.8993
	Ischaemia	3.725	26.20 (1,35)	37.6%	**0.0001**
	Age*Ischaemia	0.466	3.25 (3,35)	14.1%	**0.0323**

Members of the Popdc gene family (Popdc1, Popdc2 and Popdc3) are expressed abundantly in epithelial, skeletal, cardiac muscle and neuronal cells (Andree et al. 2000; Brand 2005). Popdc1 and Popdc2 are also abundantly expressed in cardiac pacemaker and conduction regions, and are thought to contribute to pacemaker and normal heart morphogenesis (Froese et al. 2012). Consistent with expression pattern of Popdcs, *Popdc1* and *Popdc2* were enriched in ventricular cells and cultured HL-1 cardiomyocytes, while cultured fibroblasts showed no expression of *Popdc1* and *Popdc2* (Figure 3.1). *Popdc3* is abundantly expressed in the heart, whilst cultured HL-1 cardiomyocytes and fibroblast showed no detectable expression (Figure 3.1). As discussed in Section 3.3.1, this lack of *Popdc3* expression could reflect differential expression observed from atrial vs. ventricular cells. In addition, HL-1 cells do not express certain marker proteins found in their adult atrial counterparts such as β-tubulin II, which may also be the case for *Popdc3* although this is speculative (Kuzntesov et al. 2014). Popdc1 appears vital for normal heart development as mutations in POPDC1 are associated with the congenital defect Fallot's tetralogy (Wu et al. 2013). Double Popdc1/2-deficient mice suffer sudden cardiac death when challenged with swimming stress (Froese et al. 2012). Aged (22-34-week old) wild-type animals show a similar decline in cardiac pace-making following stress-testing, which the authors suggest may be due to age-related loss of Popdc1 and Popdc2 expression in these hearts (Froese et al. 2012). The role of Popdc3 in the heart is largely unknown, although it has been shown to be down-regulated in the failing human heart (Gingold-Belfer et al. 2011).

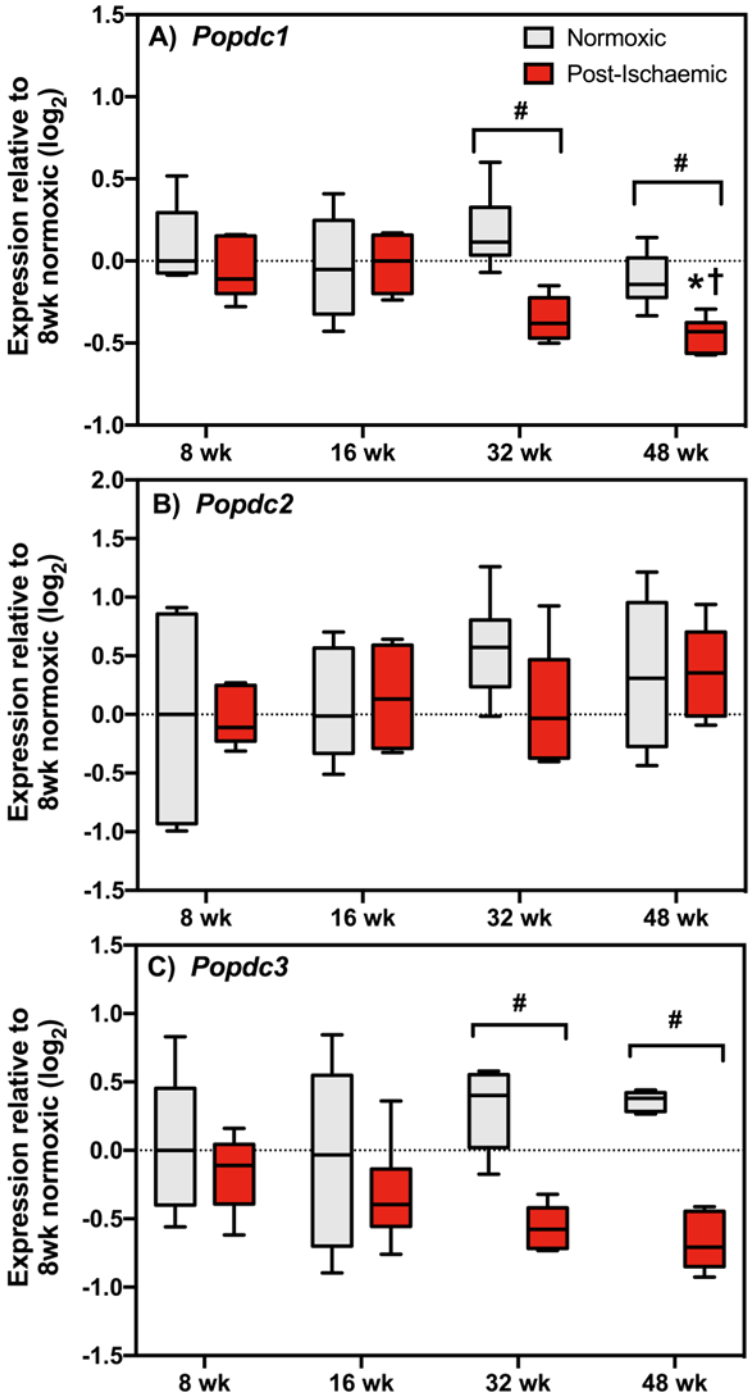


Figure 3.8 Age dependent expression of Popdc transcripts in normoxic and post-ischaemic male hearts. Shown are boxplots detailing expression changes determined by RT-qPCR for **A)** *Popdc1,* **B)** *Popdc2,* and **C)** *Popdc3* in the normoxic (grey boxes) and post-ischaemic (red boxes) heart. Boxes indicate interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values. * $P < 0.05$ vs 8-week old post-ischaemic, $P < 0.05$ † vs 16-week old post-ischaemic, # $P < 0.05$ vs age-matched post-ischaemic group (indicated by brackets). (n=6/group).

Whilst there were no changes in *Popdc1* expression in normoxic hearts, we observed a significant age-related reduction in *Popdc1* expression in 32 and 48-week old post-ischaemic hearts. Interestingly, this pattern of expression paralleled *Cav3* expression in post-ischaemic hearts. Additional to the essential role of Cav3 as a caveolae forming coat protein in cardiomyocytes, recent evidence suggest that Popdc1 is also essential for caveolae formation. Popdc1-deficient cardiomyocytes show significant reduction in caveolae with remaining caveolae being larger in size. Popdc1-deficient hearts show a loss of ischaemic tolerance leading to enhanced infarct size and impaired recovery following I-R (Alcalay et al. 2013). Furthermore, Popdc1-deficient hearts also display loss of IPC and anaesthetic preconditioning, similar to Cav3-deficient hearts (Alcalay et al. 2013; Tsutsumi et al. 2008, Tsutsumi et al. 2010). Consistent with roles for Cav3 and caveolae in countering heart failure, Popdc1 appears to be down-regulated both in murine and human end-stage failure of hearts (Gingold-Belfer et al. 2011; Feiner et al. 2011). Very little is known about the functions of Popdc2 and Popdc3 in the heart, their expression appears skeletal and cardiac muscle specific and is important for muscle differentiation and cardiac morphogenesis (Kirchmaier et al. 2012). The role of Popdc3 is largely unknown. However, reduced expression of Podpc3 is observed in the in the failing human heart and correlated with high risk and poor survival in patients with gastric cancer. (Gingold-Belfer et al. 2011; Luo et al. 2012). We observed a significant reduction in *Popdc3* expression in the post-ischaemic 48-week old hearts although what affect this could have in the aged hearts remains unclear. Combined with decrease expression of Cav3 and Cavin1 in the 32- and 48-week old post-ischaemic heart, decreased Popdc1 expression may exacerbate/accelerate loss of caveolae in the ageing post-ischaemic hearts. Thus the combined reduction of these transcripts and proteins may partially be responsible for the loss of recovery and attenuated ischaemic recovery in the 32- and 48-week old hearts.

3.4 CONCLUSION

In this study, we investigated the age-dependent expression of caveolae-related transcripts - caveolin, cavin and Popdc families in normoxic and post-ischaemic hearts from male mice. The results demonstrate significant age-dependent down-regulation of *Cav3* and *Cavin1* in both normoxic and post-ischaemic tissues. To validate transcriptional changes, Western immunoblot analysis of Cav3 and Cavin1 was performed, which confirmed the translation of gene changes to shifts in Cav3 and Cavin1 protein expression. Similar to our findings, other groups have observed age-related reductions in Cav3 and caveolae formation in hearts (Peart et al. 2014; Kidd et al. 2010), bladder smooth muscle (Lowalekar et al. 2012), and neurons (Head et al. 2010). These membrane structures and proteins are critical to stress and protective signalling as discussed previously. Ageing is associated with reduced myocardial ischaemic tolerance and many cardioprotective stimuli, including IPC and GPCR-mediated cardioprotective responses which are shown to fail in the aged hearts (Peart et al. 2014). These cardioprotective stimuli have been shown to be reliant on Cav3 and caveolae, with caveolae depletion and Cav3-deficiency shown to ablate the cardioprotective effects of these stimuli (Tsutsumi et al. 2008; Tsutsumi et al. 2010).

Current investigations of caveolin expression have employed young vs. aged or senescent models, with more intermediate levels of 'ageing' not yet studied (Park et al. 2000; Ratajczak et al. 2003; Peart et al. 2014). Similar to others, we observe that loss of ischaemic tolerance occurs before the senescent phenotype (>74 weeks old). More specifically, loss of ischaemic tolerance is apparent in 32-week old hearts and highly significant in the 48-week old hearts as shown by LDH release (Figure 3.2B). Although the 32-week old heart does not exhibit increased cell death following I-R, significant changes in developed pressure (Figure 3.2A) as a measure of functional recovery indicates that the 32-week old heart shows similar loss of recovery observed in the 48-week old hearts. Similarly, Willems and colleges (2005) have shown significant decrease in developed pressure in middle-aged to mature hearts. Coincident with these age-related changes to I-R tolerance, caveolae forming transcripts *Cav3*, *Cavin1* and *Popdc1* are down-regulated in post-ischaemic middle-aged hearts, with such combined loss hypothesized to lead to reduced caveolae formation in cardiomyocytes, leading to loss of endogenous cardioprotective responses (e.g. autacoid activation of GPCR). In support of this notion, Cav3-overexpression in the senescent heart has been shown to

restore caveolae and improve recovery (Kidd et al. 2010). This further suggests an age-related decline of caveolae may partially contribute to loss of ischaemic tolerance in these hearts. Finally, Cavin1 and Popdc1 have recently also been shown to be critical for formation of caveolae in cardiomyocytes (Alcalay et al. 2013; Kasahara et al. 2014). Thus, the age-dependent loss of Popdc1 and Cavin1 in the post-ischaemic heart, combined with loss of Cav3, may contribute to depletion of caveolae (and impaired stress signalling/resistance). There was also a notable decline in *Popdc3* expression in 32 and 48 week old hearts, although its impact on the ageing heart is unknown as the role of Popdc3 remains uncharacterised. Given their homology to each other is only 25%, the role of *Popdc3* may be substantially different to that of *Popdc1* (Andree et al. 2000). Taken together, age-related loss of caveolae-forming transcripts *Cav3*, C*avin1* and *Popdc1* may result in reduction of caveolae, which may partially contribute to loss of ischaemic tolerance and cardioprotective efficacies in the middle-aged heart.

4. Chapter Four

Effects of age on caveolae-related transcript expression in normoxic and post-ischaemic female hearts

ADDENDUM

Contributions to Chapter 4:

Can Kiessling:

- Experimental design
- All laboratory bench-work
- Data analysis
- Author of the chapter

Kevin Ashton:

- Assistance with experimental design and interpretation of results

Melissa Reichelt:

- Heart perfusions

Selected data from this chapter have been previously published

Kiessling C.J., Reichelt M.E., Peart, J., Headrick J.P., Ashton K.J. (2014). Transcriptional Analysis of Caveolin and Cavin in the Male and Female Ageing Mouse Heart Following Ischaemic Stress. Circulation Research. 115: **A220**

4.1 INTRODUCTION

Ischaemic heart disease (IHD) is the leading cause of death in Australia for both males and females (ABS 2014). Epidemiological studies have suggested that prevalence and mortality from IHD in males is twice that when compared to females at all ages, with females one-third as likely as males to report a heart attack (AIHW 2010). Women develop IHD on average 10 years later than men, with myocardial infarction occurring 20 years later (Duvall 2003; Bassuk & Manson 2010). However, when post-menopausal females are compared to age-matched males this protective effect is shown to be reversed, as there is a 10-fold increase in IHD after menopause compared with only a 4.6-fold increase in aged-matched males (Korzick & Lancester 2013; Duvall 2003). Several factors have been suggested to contribute to development of IHD in women, including traditional Framingham risk factors such as hyperlipidaemia, hypertension, smoking and psychosocial factors including mental stress and anxiety (Mehta, Wei & Wenger 2015). For over 90% of Australian women, there is at least one modifiable risk factor for CVD, whilst half of all women have two or three (AIHW 2010). These modifiable risk factors include low fruit and vegetable consumption, being overweight or obese, high blood cholesterol and physically inactive (AIHW 2005).

Circulating estrogen and estrogen receptor (ER) activation have been implicated in the current paradigm of sex-related differences in cardioprotection. Indeed, animal models support the role of circulating estrogen in reducing atherosclerosis as ovariectomy results in increased formation of atherosclerotic plaques whilst estrogen supplement reverses this effect on the aorta (Mayer et al. 2005). Plasma estradiol (E2) concentrations in postmenopausal women have been reported to be 2.5x to 25x less compared to the cyclic variation observed in premenopausal women (Bremnes et al. 2006; Sullivan et al. 1995). Indeed, estrogen replacement therapy has been suggested to reduce the occurrence of IHD by nearly 50% in treated women compared with non-users (Rosano & Fini 2002). Furthermore, ovariectomy in mice results in increased infarct sizes in both young and senescent female hearts, further highlighting a role for estrogen (Hunter et al. 2007). However, estrogen signalling in itself is not sufficient to explain the sex-related differences, as there is a lack of efficacy of hormone replacement therapy in prevention of cardiovascular disease in pre- and post-menopausal women (Rossouw et al. 2002; Rossouw et al. 2007).

The exact mechanisms of this protection in the female heart is unknown and is thought to involve multiple estrogen GPCRs, including estrogen receptor-α (ERα), estrogen receptor-β (ERβ) and G-protein receptor 30 (GPR30). Activation of ERs leads to transcriptional and non-transcriptional effectors (Ostadal & Ostadal 2014). Transcriptional effectors have been shown to include increased eNOS and L-type Ca^{2+} channel gene expression which may be responsible for the reduced cell death and increased functional recovery observed in female hearts (Murphy 2011; Babiker et al. 2002). Whilst non-transcriptional changes include enhanced calcium handling, protein S-nitroyslation, phosphorylation status of survival kinases and enhanced mitochondrial survival following I-R in the female myocardium (Deschamps, Murphy & Sun 2010). Both acute and chronic estrogen treatment in animal models has been shown to enhance dilator sensitivity of the coronary microcirculation through enhanced NO production by the endothelium as well as reducing no-reflow zones following I-R (Thompson, Pinkas & Weiner 2000; McCullough et al. 2001). Furthermore, estrogen replacement in aged (24-month old) female rats reverses the age-related impairment of flow-induced dilation in coronary arterioles by approximately 160% (LeBlanc et al. 2009).

Sex-related differences in cardiomyocytes may partially contribute to the differences in response to disease as they have notable differences in electrophysiology, contractility, signalling, metabolism, and cardioprotection (Murphy & Steenbergen 2014). Experimentally, the young female myocardium has been shown to be more resistant to variety of stressors such as I-R damage and associated arrhythmias, stress-induced hypertrophy and heart failure (Bell et al. 2013). Generally, disruption of a gene results in exacerbation of I-R injury in males when compared to female counterparts. For example, β_2-adrenergic receptor and Ncx-deficiency has been shown to decrease I-R tolerance in male but not female mice (Cross et al. 2002; Cross et al. 1998). There are also notable differences in cardioprotective mediator response as well. For example, female cardiomyocytes have increased eNOS association with Cav3, which is thought to enhance *S*-nitroyslation of the L-type Ca^{2+} channel in females (Sun et al. 2006). Furthermore, caveolae-derived eNOS has also been shown to enhance *S*-nitroyslation of mitochondrial proteins although this remains to be shown for females (Sun et al. 2015). Unlike their male counterparts, some of this protection is also retained with ageing (Willems et al. 2005).

Ageing studies investigating sex-related differences in cardioprotection in mice have predominantly used the aged phenotype (>70 weeks old) (see Table 1 of Boengler & Shulz 2009). However, the reduction in ischaemic tolerance can be seen well before the aged phenotype in both sexes (Willems et al. 2005). As discussed in Chapter 3 the exact mechanisms behind the loss of cardioprotective signalling in the ageing heart is unknown. Furthermore, sex-related differences have not investigated the expression of caveolae coat proteins which are also crucial determinants of endogenous cardioprotection. As previously discussed, ablation of caveolae results in loss of ischaemic tolerance, whilst overexpression results in restoration of ischaemic tolerance in the aged hearts (Tsutsumi et al. 2010; Kidd et al. 2010). Changes in Cav3-eNOS association following I-R have also been shown to be a key sex-related difference (Sun et al. 2006). However, the expression of caveolins, cavins and Popdcs remain largely uncharacterised in the female heart, whose differential expression may also contribute to sex-related differences with ageing. We therefore sought to investigate the expression of caveolae-related transcripts in the female normoxic and post-ischaemic ageing myocardium in a similar fashion to Chapter 3.

4.2 METHODS

Langendorff perfusions were carried out on hearts (n=8-10/per group) from 8, 16, 32 and 48 week old female mice as previously described in Section 2.1.1. Briefly, 20 minutes of global normothermic ischaemia was followed by 60 minutes of reperfusion whilst normoxic hearts were reperfused for 80 minutes.

RNA isolation and cDNA synthesis from normoxic and post-ischaemic left ventricular tissue was carried out as described in Section 2.2.1.

RT-qPCR was used to analyse expression of caveolin, cavin and Popdc transcripts in ageing hearts as detailed in Section 2.3.1. Selection of a stable reference gene was carried out according to Section 2.3.2. Normalisation of post-ischaemic gene expression to normoxia was carried out according to Section 2.4.

Western immunoblotting was carried to confirm validate the relationship between transcript and protein expression changes for Cav3 and Cavin1 according to Section 2.5.

Statistical analysis was conducted on data as per Section 2.6 with statistical significance accepted for $P < 0.05$.

4.3 RESULTS AND DISCUSSION

4.3.1 Functional and oncotic indicators of myocardial injury in post-Ischaemic ageing female mouse heart

Age-related reduction in functional recovery became apparent in the 32- and 48-week old female heart when compared to their 8 and 16-week old counterparts (Figure 4.1A). A similar reduction in functional recovery was observed in the 32- and 48-week old male hearts (Chapter 3, Figure 3.2A). This reduction in functional recovery of the female 32-week old heart was not observed by other groups using similar ageing time points and I-R methods (Willems et al. 2005). Their results suggested that the reduction in functional recovery is initially detected in 48-week old female hearts whilst we observed this in 32-week old hearts. Interestingly, the reduction in post-ischaemic recovery in the 32-week old hearts did not correlate with enhanced LDH release (Figure 4.1B). Significant LDH release was present only in the 48-week old female hearts vs. 8-week old female hearts (Figure 4.1B), similar to male counterparts (Figure 4.1B). In contrast to Willems et al. (2005) who showed that female 48-week old have significantly lower LDH release when compared to male counterparts, our findings demonstrated the opposite trend (Figure 4.1B).

As previously discussed the sex-related differences in humans is often attributed to estrogen signalling during pre-menopause. It is difficult to assess whether the loss of ischaemic tolerance observed in the 32- and 48-week old female hearts is associated with a decline in these protective mechanisms in female hearts. The time frame and duration of menopause is unknown and difficult to assess in animal models. However, the reproductive capacity of female mice declines after approximately 8 months (34 weeks) as female mice are retired from breeding at 8 months because litter size diminishes (Jackson Laboratory 2011). Furthermore, rapid deterioration of the follicle reserve has been observed between 48-72 weeks of age (Niikura, Niikura & Tilly 2009). It is therefore tempting to speculate that the 32- and 48-week old mice may be having commenced perimenopause and this may be responsible for the significant oncosis present in the 48-week old hearts and reduced recovery we observed in the 32- and 48-week old hearts used in this study.

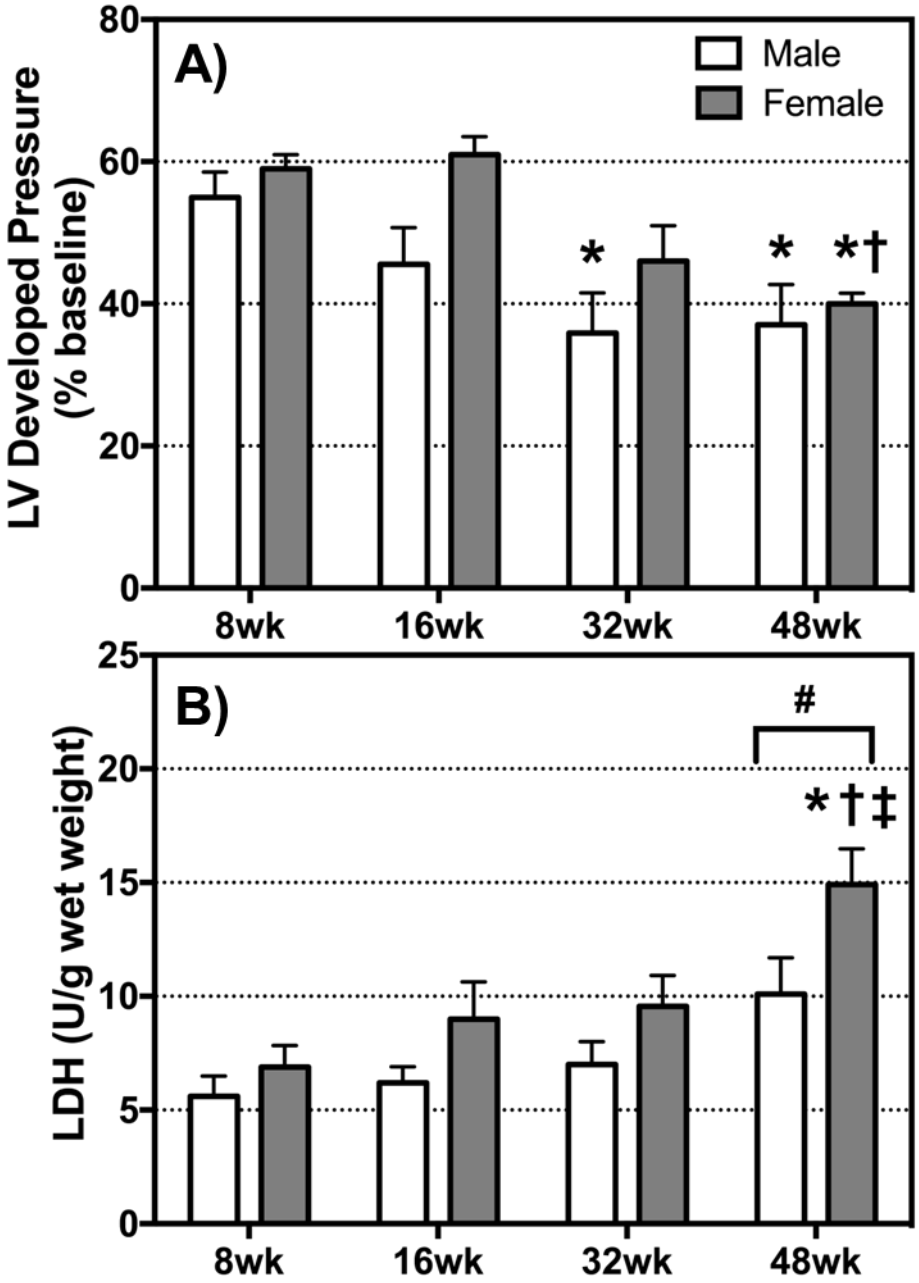


Figure 4.1 Recovery from I-R in the ageing female heart. Data are shown for **A)** developed pressure and **B)** cell death following 20 min ischaemia and 60 min reperfusion in isolated hearts from 8, 16, 32 and 48-week old male (white boxes) and female (grey boxes) mice. Data are expressed as mean±SEM; **P* < 0.05 8-week old female; †*P* < 0.05 vs 16-week old female, ‡ P < 0.05, vs 32-week old female; # *P* < 0.05 male vs female age-matched group (indicated by brackets). (n=6-8/group).

4.3.2 Reference gene stability in the ageing normoxic and post-ischaemic hearts

Determination of a stable reference gene was carried out in an identical to the previous male study (Section 3.3.3). As shown in Figure 4.2 *Ppia*, *Actb*, *Pgk1* and *Rpl13*a were all considered stably expressed in both the normoxic and post-ischaemic groups, with minimal difference in stability for all four genes. Although *Ppia* was identified as the most stably expressed gene, *Pgk1* was selected to allow for more uniform cross comparisons and consistency with the findings from the male study in Chapter 3. However, there were large differences in male vs female *Pgk1* expression (3.26-fold) which prohibited direct across-sex comparisons as previously detailed in Section 2.3.3.

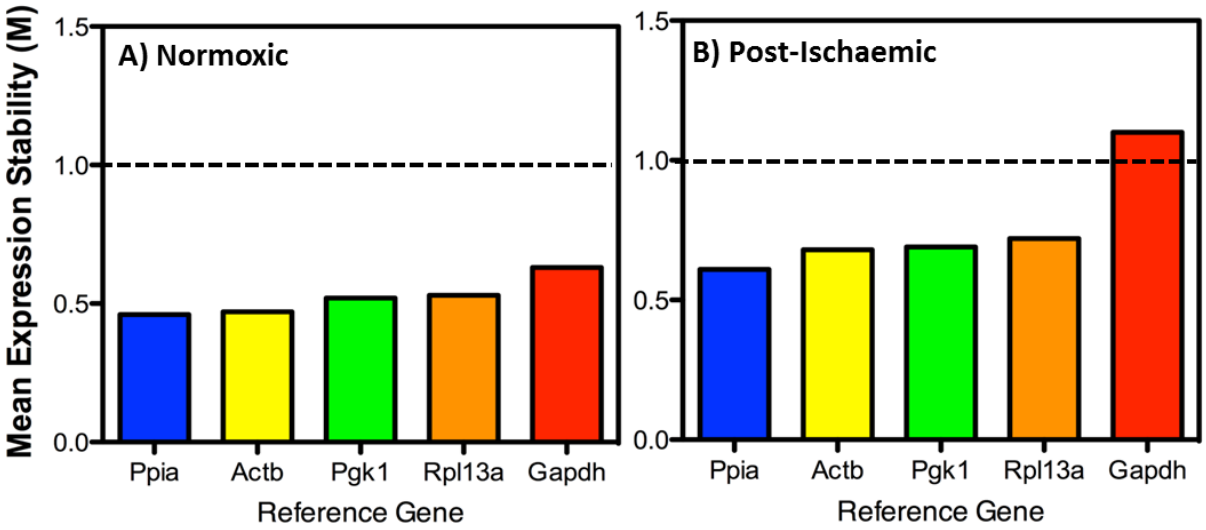


Figure 4.2 Expression stability values (M) of the five putative reference genes (*Actb, Gapdh, Pgk1, PPia* and *Rpl13a*) tested in the female **A)** normoxic ageing hearts and **B)** post-ischaemic ageing hearts (n=24/group). The dotted line indicates the recommended threshold value of <1.0 for heterogeneous samples.

4.3.3.1 Transcriptional analysis of caveolin expression

Similar to the male study (as detailed in Chapter 3), RT-qPCR was used to study expression of caveolin, cavin and Popdc transcripts in normoxic and post-ischaemic ageing female hearts. *Cav1* showed a 1.6-fold down-regulation that was significant in the 48-week old hearts when compared to 32-week old normoxic hearts (Figure 4.3A). With male hearts demonstrating a similar down-regulation of *Cav1* at 48-weeks old (Figure 3.4A). Whilst there were notable perturbations to post-ischaemic *Cav1*, its expression returned to baseline levels in the middle-aged hearts (Figure 4.3A). However, Two-way ANOVAs on expression levels of *Cav1* revealed significant effects of age and ischaemia (Table 4.1). This can be visualised in the 48-week old post-ischaemic hearts which showed induction (2.0-fold) of *Cav1* when compared to age-matched normoxic counterparts (Table 4.1) (Figure 4.3A), similar to that of males. Normoxic *Cav2* showed no changes in expression with ageing (Figure 4.3B) while male counterparts showed significant down-regulation in the 48-week old normoxic hearts (Figure 3.4B). Analysis of *Cav2* expression yielded a significant main effect for both age and ischaemia (Table 4.1). More specifically, *Cav2* showed significant induction in the post-ischaemic 32- and 48-week old hearts when compared to 16-week old hearts (Figure 4.3B). In addition, significant increase in *Cav2* transcript expression was present in the 32- and 48-week old post-ischaemic hearts vs age-matched counterparts (Figure 4.3B) which was not observed in the males.

A non-significant minor trend in *Cav3* down-regulation was observed in the ageing normoxic heart as early as 16-weeks of age (Figure 4.3C). This is similar to expression patterns observed in the male normoxic heart (Figure 3.4C). Similar to male counterparts, analysis of *Cav3* expression yielded a significant main effect for both age and ischaemia although the interaction between age and ischaemic was borderline non-significant (P=0.051) (Table 3.1). While *Cav3* was down-regulated in the 16-week old post-ischaemic hearts when compared to 8-week old hearts, the 32- and 48-week old hearts displayed 2.0-fold up-regulation when compared to 16-week old post-ischaemic hearts (Figure 4.3C). *Cav3* mRNA showed significant over-expression (2.0-fold) in the 8-, 32- and 48-week old post-ischaemic female hearts when compared to their age-matched normoxic counterparts (Figure 4.5C). Male hearts previously demonstrated a significant up-regulation of *Cav3* only in the 16- and 32-week old post-ischaemic heart (Figure 3.4C). Therefore, the induction of *Cav3* mRNA following I-R is present in both males and females although they differ in the patterns of expression with ageing.

Table 4.1 Two-way analysis of variance of age and post-ischaemic effects on caveolin transcript expression in the female heart

Gene	Factor	Mean Square	F (DFn, DFd)	% of Total Variation	*P* value
Cav1	Age	1.210	4.36 (3,38)	20.0%	**0.0098**
	Ischaemia	0.804	2.90 (1,38)	4.4%	0.0967
	Age*Ischaemia	0.998	3.60 (3,38)	16.5%	**0.0220**
Cav2	Age	0.826	3.62 (3,40)	13.1%	**0.0211**
	Ischaemia	4.820	21.21 (1,40)	25.4%	**< 0.0001**
	Age*Ischaemia	0.847	3.71 (3,40)	13.4%	**0.0192**
Cav3	Age	1.397	6.91 (3,38)	19.7%	**0.0008**
	Ischaemia	8.158	40.32 (1,38)	38.3%	**< 0.0001**
	Age*Ischaemia	0.571	2.82 (3,38)	8.1%	0.0516

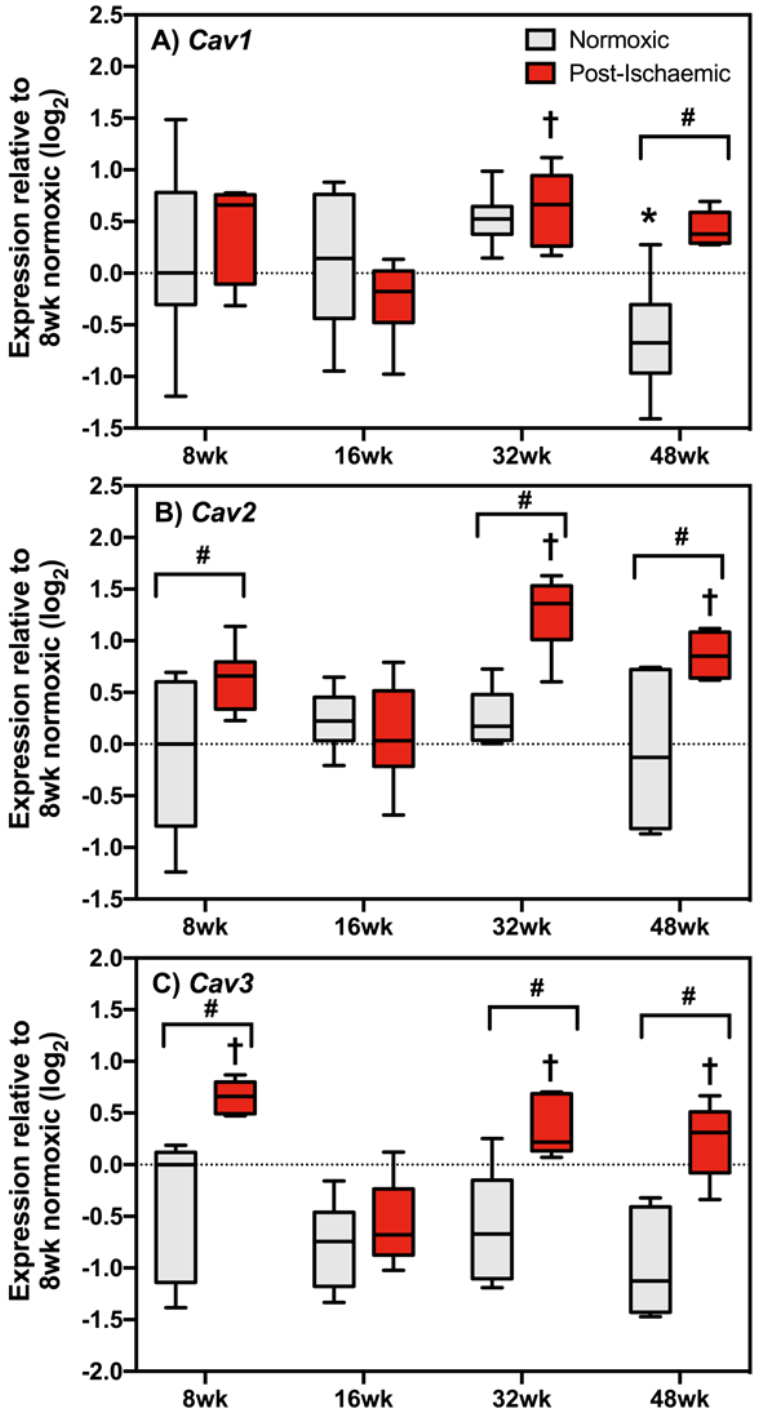


Figure 4.3 Age dependent expression of caveolin transcripts in normoxic and post-ischaemic female hearts. Shown are boxplots detailing expression changes determined by RT-qPCR for **A)** *Cav1,* **B)** *Cav2* and **C)** *Cav3* in the normoxic (grey boxes) and post-ischaemic (red boxes) heart. Boxes indicate interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values. * $P < 0.05$ vs 32-week old normoxic; † $P < 0.05$ vs 16-week old post-ischaemic; # $P < 0.05$ vs age-matched post-ischaemic group (indicated by brackets). (n=6/group).

4.3.3.2 Immunoblot analysis of caveolin3 protein expression

To verify the relationship between *Cav3* transcript and protein expression changes immunoblotting was used. In the normoxic 48-week old female heart Cav3 showed a significant 1.6-fold reduction in protein expression (Figure 4.4A). This finding was similar to observations in the male normoxic heart, which demonstrated a significant age-related reduction (1.5-fold) in Cav3 (Figure 3.5A). In the post-ischaemic 48-week old female heart, Cav3 protein expression was down-regulated 1.7-fold (P=0.07) relative to 8-week old hearts (Figure 4.4B). In the male counterparts, Cav3 expression was significantly reduced in both the 48-week old normoxic and post-ischaemic hearts (Figure 3.5A & Figure 3.5B). Taken together, there is an age-related reduction in Cav3 in both the normoxic and post-ischaemic male and female hearts.

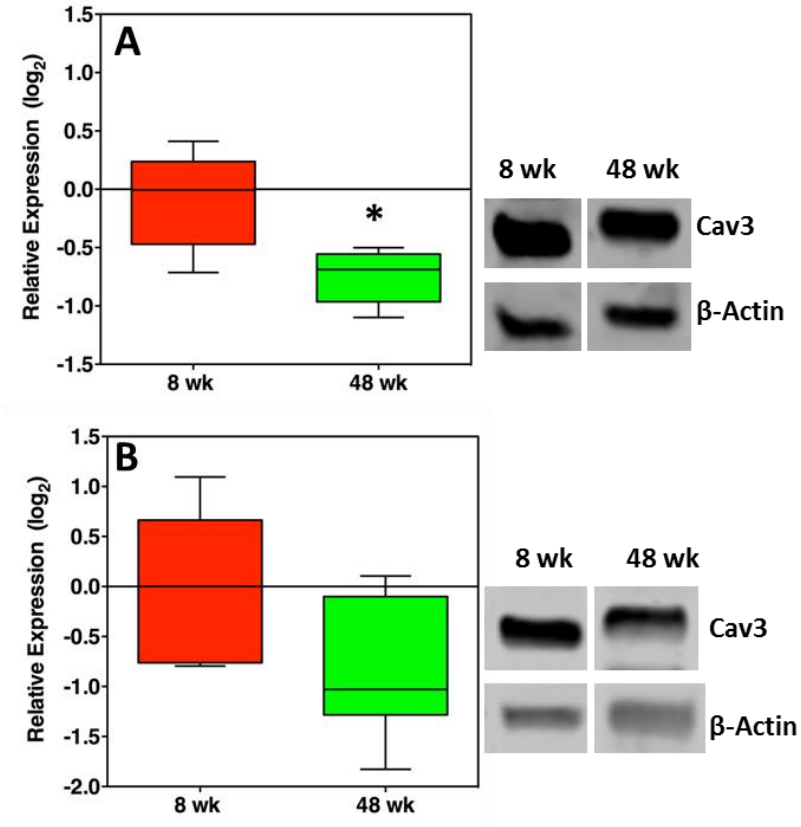


Figure 4.4 Age-dependent Cav3 protein expression in normoxic and post-ischaemic female hearts. Boxplots are shown detailing Cav3 protein expression in **A)** normoxic and **B)** post-ischaemic ageing hearts. Boxes indicate the interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum. **P* < 0.05 *vs.* 8-week old. (n=6/group).

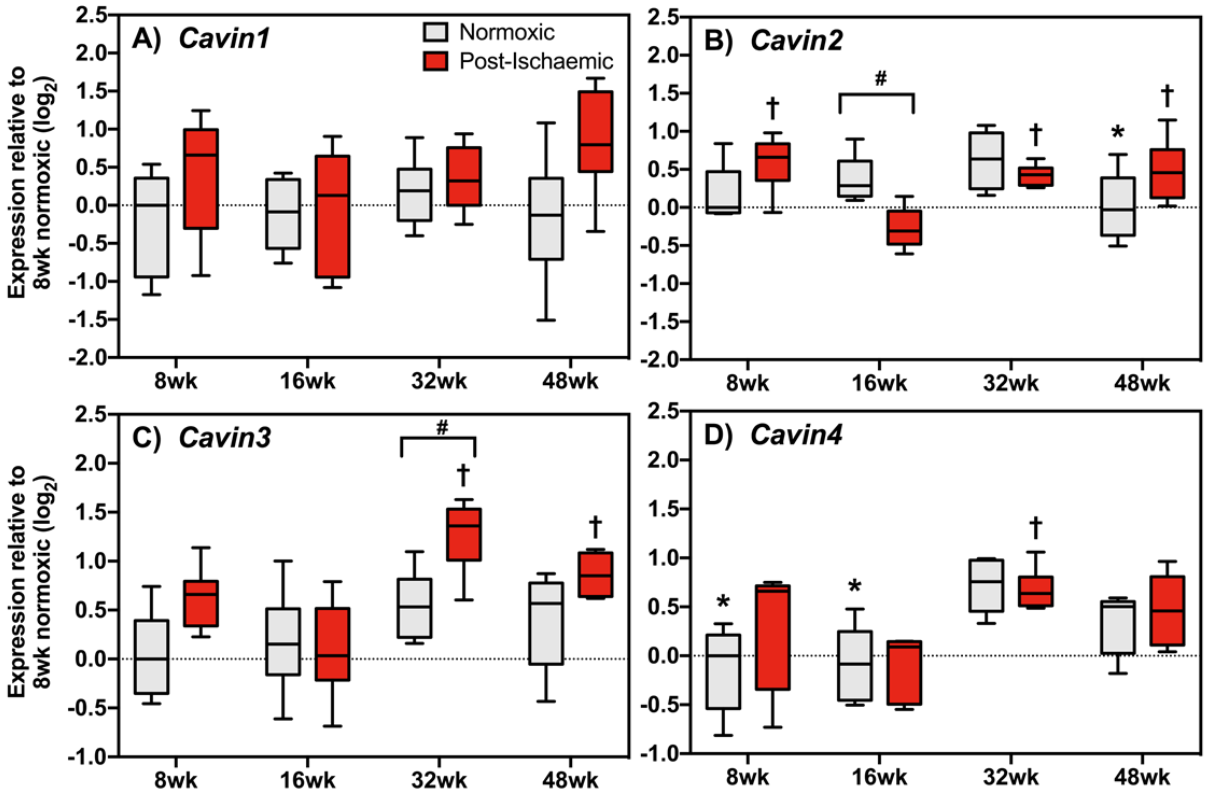
4.3.3.3 Transcriptional analysis of cavin expression in the ageing female mouse normoxic and post-ischaemic female mouse heart

In the normoxic female heart, *Cavin1* showed no changes in expression with ageing and did not show significant interaction of age and ischaemia (Table 4.2, Figure 4.5A). Expression levels were however different in the male heart, where *Cavin1* showed significant down-regulation in the 16- and 48-week old normoxic heart (Figure 3.6A) and showed significant interaction of age. In the post-ischaemic female heart, *Cavin1* demonstrated no age-related changes in transcript expression (Figure 4.7A) whilst male counterparts showed significant down-regulation the in 16 and 48-week old hearts (Figure 3.6A). Although the post-ischaemic female hearts did not show age-related changes compared to their normoxic counterparts, the 48-week old post-ischaemic heart showed nearly 2-fold up-regulation of *Cavin1* that was close to statistical significance (P= 0.05) (Figure 4.5A). Expression of *Cavin2* in the ageing normoxic and post-ischaemic female hearts was largely stable although there were minor significant differences were detected (Figure 4.5B). Post-ischaemic male hearts previously demonstrated significant down-regulation of *Cavin2* in the 48-week old heart vs 8-week (Figure 3.6B). A significant interaction of age and ischaemia was detected for *Cavin2*; this was only significant in the 16-week old hearts which displayed a 1.5-fold reduction in *Cavin2* when compared to normoxic counterparts. Whereas male counterparts displayed a reduction in the 16-week old and older hearts.

Normoxic *Cavin3* did not show significant changes with ageing (Figure 4.5C), this was similar to male hearts. Significant up-regulation of *Cavin3* was present in the 32- and 48-week old post-ischaemic heart compared to 16-week old (Figure 4.5C) with this interaction of age an ischaemia supported by two-way ANOVA analysis (Table 4.2). This induction of *Cavin3* in the 32-week old hearts was also significant when compared to their-age matched normoxic counterparts (Figure 4.5) and was not present in males. In the normoxic ageing hearts, *Cavin4* showed significant 1.6-fold up-regulation the 32-week old hearts when compared to 8- and 16-week old hearts (Figure 4.5C). Post-ischaemic expression of *Cavin4* showed minor changes in expression with ageing and returned to baseline levels (Figure 4.5D) similar to males.

Table 4.2 Two-way analysis of variance of age and post-ischaemic effects on cavin transcript expression in the female heart

Gene	Factor	Mean Square	F (DFn, DFd)	% of Total Variation	*P* value
Cavin1	Age	0.385	0.84 (3,38)	5.1%	0.4816
	Ischaemia	2.511	5.46 (1,38)	11.0%	**0.0248**
	Age*Ischaemia	0.533	1.16 (3,38)	7.0%	0.3377
Cavin2	Age	0.482	4.00 (3,40)	16.5%	**0.0038**
	Ischaemia	0.000	0.01 (1,40)	0.1%	**< 0.0001**
	Age*Ischaemia	0.842	6.99 (3,40)	28.8%	**0.0017**
Cavin3	Age	1.393	8.07 (3,39)	29.8%	**0.0003**
	Ischaemia	2.127	12.33 (1,39)	15.2%	**0.0011**
	Age*Ischaemia	0.376	2.18 (3,39)	8.1%	0.1058
Cavin4	Age	1.436	9.92 (3,40)	40.2%	**< 0.0001**
	Ischaemia	0.189	1.31 (1,40)	1.8%	0.2594
	Age*Ischaemia	0.141	0.97 (3,40)	4.0%	0.4142



4.5 Age dependent expression of cavin transcripts in normoxic and post-ischaemic female hearts. Shown are boxplots detailing expression changes determined by RT-qPCR for **A)** *Cavin1,* **B)** *Cavin2,* **C)** *Cavin3* and **D)** *Cavin4* in the normoxic (grey boxes) and post-ischaemic (red boxes) heart (n=6/group). Boxes indicate interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values. * $P < 0.05$ vs 32-week old normoxic; † $P < 0.05$ vs 16-week old post-ischaemic, # $P < 0.05$ vs age-matched post-ischaemic group (indicated by brackets). (n=6/group).

4.3.3.4 Immunoblot Cavin1

In agreement with the transcriptional analysis, Cavin1 protein did not show an age-related change in expression in the normoxic female heart (Figure 4.6A). In the male normoxic heart a minor trend in down-regulation of Cavin1 (although not statistically significant) was observed. In post-ischaemic female hearts, transcriptional analysis suggested no significant age-related changes in *Cavin1* expression were present. However proteomic analysis of Cavin1 showed significant 1.6-fold decrease (Figure 4.6B). Similarly, in the male post-ischaemic counterpart, there was a 1.6-fold reduction in Cavin1 protein expression in the 48-week old heart. Collectively the 48-week old male and female post-ischaemic myocardium display loss of Cavin1, although this finding was unexpected in the female 48-week old hearts as transcriptional analysis suggested no transcriptional changes.

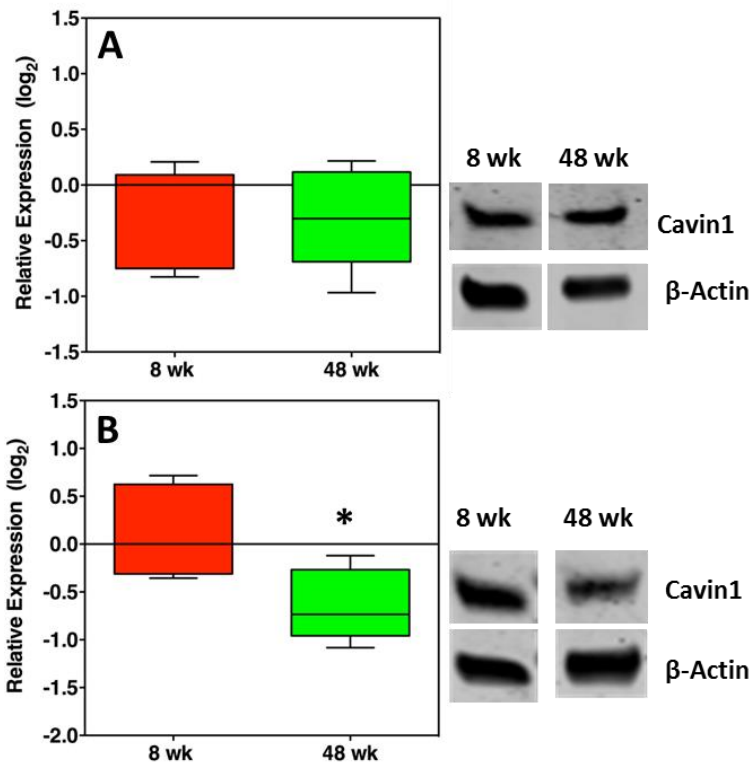


Figure 4.6 Age-dependent expression of Cavin1 protein in normoxic and post-ischaemic female hearts. Boxplots are shown detailing Cavin1 protein expression in **A)** normoxic and **B)** post-ischaemic ageing hearts. Boxes indicate the interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values. To the right of the graphs representative immunoblot images are shown for cavin1 and the β-Actin loading control. *$P < 0.05$ *vs.* 8-week old. (n=6/group).

4.3.3.5 Transcriptional analysis of Popdc expression in the ageing male mouse normoxic and post-ischaemic female mouse heart

A general up-regulated pattern with increased age was observed for *Popdc1* similar to male normoxic counterparts. In the post-ischaemic female hearts, *Popdc1* showed a significant down-regulation (2.0-fold) in 16-week old hearts when compared to all other groups (Figure 4.7A). This interaction of age and ischaemia is also supported by two-way analysis (Table 4.3). Whilst male post-ischaemic ageing hearts showed repression of *Popdc1* in the 32 and 48-week old hearts (Figure 3.6A). Down-regulation of *Popdc1* was only present in the 16-week old post-ischaemic hearts vs age-matched normoxic hearts (Figure 4.7A). Two-way ANOVAs on expression levels of *Popdc1* revealed significant effects of ischaemia and ageing (Table 4.3) although this was largely unaffected with ageing as shown in figure 4.7A. Whilst male counterparts previously showed down-regulation in the 32- and 48-week old post-ischaemic hearts vs age-matched normoxics (Figure 3.8A). *Popdc2* demonstrated no changes with ageing (Figure 3.8B) similar to males. Normoxic and post-ischaemic *Popdc3* cardiac expression showed no changes (Figure 4.7C). Male counterparts previously demonstrated repression of *Popdc3* in 32 and 48-week old post-ischaemic hearts. Interestingly, when compared to their normoxic counterparts, *Popdc3* showed induction with ageing, significant in the 8, 32 and 48-week old hearts (Figure 4.7C). With this main effect of ischaemia also supported by two-way ANOVA analysis (Table 4.3). This was in contrast to male counterparts, which showed significant *Popdc3* reduction in the 32 and 48-week old post-ischaemic hearts vs normoxic counterparts (Figure 3.8C).

Table 4.3 Two-way analysis of variance of age and post-ischaemic effects on Popdc transcript expression in the female heart

Gene	Factor	Mean Square	F (DFn, DFd)	% of Total Variation	*P* value
Popdc1	Age	1.335	3.63 (3,40)	14.4%	**0.0208**
	Ischaemia	2.307	6.28 (1,40)	8.3%	**0.0164**
	Age*Ischaemia	2.256	6.14 (3,40)	24.4%	**0.0016**
Popdc2	Age	1.204	4.78 (3,40)	24.7%	**0.0063**
	Ischaemia	0.091	0.36 (1,38)	0.6%	0.5498
	Age*Ischaemia	0.464	1.85 (3,38)	9.5%	0.1555
Popdc3	Age	0.877	6.91 (3,40)	18.9%	**0.0007**
	Ischaemia	5.798	45.69 (1,40)	41.7%	**< 0.0001**
	Age*Ischaemia	0.133	1.05 (3,40)	2.9%	0.3806

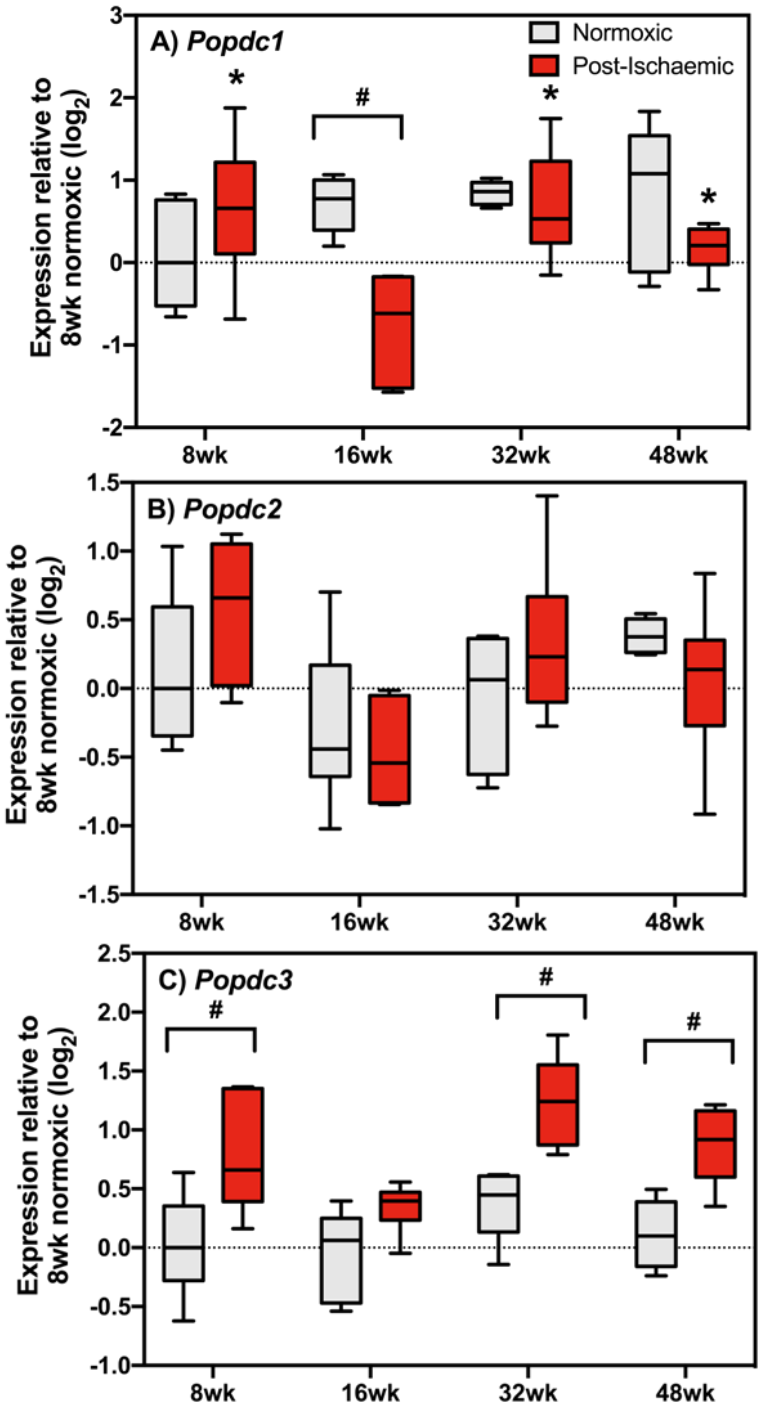


Figure 4.7 Age dependent expression of Popdc transcripts in normoxic and post-ischaemic female hearts. Shown are boxplots detailing expression changes determined by RT-qPCR for **A)** *Popdc1,* **B)** *Popdc2,* and **C)** *Popdc3* in the normoxic (grey boxes) and post-ischaemic (red boxes) heart. Boxes indicate interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values. * $P < 0.05$ vs 16-week old post-ischaemic, # $P < 0.05$ vs age-matched post-ischaemic group (indicated by brackets). (n=6/group).

Ageing is associated with increased prevalence of IHD; however, females appear to show a reduced prevalence of IHD when compared to age-matched males until after 75 years of age (Mosca Barrett-Connor & Wenger 2011). Many studies implicate ER mediated signalling as a key contributor in the sex-based differences in cardioprotective signalling, as attenuation of cardioprotection is correlated with menopause (see review Yang & Reckelhoff 2011). Sex-based studies have largely focused on ERα, ERβ and GPR30 as they are expressed in both the male and female left ventricular tissue. Interestingly, female hearts show higher expression levels of ERs relative to males (Patterson et al. 2010; Filice et al. 2009). Whilst activation of these receptors is cardioprotective there is conflicting evidence as to whether they are caveolae-dependant. More specifically, although ERα has been shown to localise in endothelial caveolae, conflicting reports exist whether Cav3 and ERα co-localise in cardiomyocytes (Chambliss et al. 2000; Chung et al. 2009; Mahmoodzadeh et al. 2006). These differences may arise from different cardiomyocyte cell models used, specifically neonatal cardiomyocytes compared to adult cells. Thus far only one sex-related study has shown differential interaction of Cav3 with eNOS following I-R (Sun et al. 2006). However, the expression of caveolae coat proteins remains uncharacterised in the ageing female heart, which may contribute to the sex-related differences in cardioprotection. Thus, in a similar manner to Chapter 3, we sought to characterise the expression of caveolae coat proteins including members of caveolin, cavin and Popdc genes in an attempt to investigate sex-based differences. We hypothesised that the female aging myocardium would not show the age-related loss of caveolae coat proteins observed in the male myocardium. Surprisingly, we observed the down-regulation of several crucial caveolae-related transcripts and proteins similar to their male counterparts.

Transcriptional analysis of *Cav3* in normoxic female hearts showed a minor down-regulated trend in *Cav3* as early as 16-week old hearts, similar to expression patterns observed in male counterparts. However, proteomic validation showed that the non-significant mRNA reduction in *Cav3* in the normoxic hearts was significant at the protein level in the 48-week old hearts (Figure 4.4A). Conversely the post-ischaemic hearts suggested significant down-regulation of *Cav3* at the transcriptional level as early as 16-weeks old (Figure 4.4C), although proteomic validation of Cav3 in the 48-week old heart showed a borderline non-significant reduction (P=0.07; Figure 4.5B). However, given the combined reduction of *Cav3*, *Cavin1* and

Popdc1 in the ageing post-ischaemic heart, it is possible that caveolae formation in these hearts is significantly reduced although this remains to be shown morphologically (e.g. by electron microscopy) and warrants future investigation. Taken together, the post-ischaemic female ageing heart shows similar repression of several caveolae-essential coat proteins which may impair caveolae formation in the female myocardium. As caveolae forming transcripts *Cav3* and *Popdc1* are down-regulated in the middle-aged hearts, as well as Cavin1 protein, this may lead to a reduction in cardiac caveolae formation thereby may be partially responsible for the loss of ischaemic tolerance in the middle-aged hearts. Interestingly, the LDH release observed in the 16- and 32-week old hearts parallels the expression of caveolae forming transcripts *Cav3* (Figure 4.4C) and *Popdc1* (Figure 4.7A), thus suggesting non-caveolae mechanisms may be responsible for protection observed in these hearts.

There is conflicting evidence regarding the localisation of ERs in cardiomyocyte caveolae (Chung et al. 2009; Mahmoodzadeh et al. 2006). It is possible these differences may be species-related (mouse vs. human). If ERs are not enriched in caveolae then ER signalling may be preserved in the ageing female heart, thus leading to enhanced recovery even if caveolae density is reduced. Indeed, acute ER-α activation has been shown to restore ischaemic tolerance in the senescent (>74 week old) female mouse heart and has been suggested as a therapeutic in aging women with IHD (Novotny et al. 2009). In addition, activation of the cardioprotective GPR30 may also be responsible for I-R tolerance in these hearts and whether it selectively partitions in cardiomyocyte caveolae is unknown (Deschamps & Murphy 2009).

4.4 CONCLUSION

In this study, we sought to investigate the expression of caveolae-related transcripts in the ageing female heart and whether changes in expression correlated with enhanced cardioprotection. However, at the functional level the ageing female heart showed a similar loss of ischaemic tolerance comparable to the age-matched male myocardium (Chapter 3). More specifically, the protected phenotype observed in mature to middle-aged female hearts (32- and 48-weeks) as reported in Willems and colleagues (2005) was not observed. The reasons for this discrepancy are unknown as the strain of mouse and I-R protocols are similar between studies. We also observed down-regulation of caveolae transcripts similar to that observed in male counterparts, which also coincided with the loss of endogenous ischaemic tolerance in the middle-aged hearts. Notably, in the normoxic ageing heart *Cav3* showed significant down-regulation as early as 16-weeks old. Transcriptional and proteomic analysis ($p=0.07$) of Cav3 demonstrates repression in the 48-week old heart. Recently, Cavin1 and Popdc1 have also been shown to be crucial for cardiomyocyte caveolae formation (Kasahara et al. 2014; Alcalay et al. 2013). Whilst Cavin1 expression did not change with aging in the normoxic heart, the 48-week old post-ischaemic heart showed significant decrease of Cavin1, which was confirmed by immunoblotting. The repression of these transcripts and their proteins coincide with the loss of cardioprotection in these hearts and may be partially responsible for the loss of endogenous protection. There are conflicting reports about the caveolae localisation of ER-α localisation in the heart (Chung et al. 2009; Mahmoodzadeh et al. 2006). If indeed ER-α does not localise in caveolae, then it is possible ER-mediated protection is caveolae/Cav3-independent and may be responsible for the protection observed in the female 16- and 32-week old post-ischaemic hearts. Taken together, the combined reduction of *Cav3, Cavin1* and *Popdc1* in the middle-aged female myocardium may lead to a reduction in caveolae density and subsequently responsible for the loss of cardioprotection observed in the middle-aged post-ischaemic hearts. Future studies should examine whether the age-related reductions of these transcripts result in morphological loss of caveolae in the middle-aged female hearts.

5. Chapter Five

RNA interference of Caveolin-3 in the HL-1 cardiomyocyte

ADDENDUM

Contributions to Chapter 5:

Can Kiessling:

- Experimental design
- All laboratory bench-work
- Data analysis
- Author of the chapter

Kevin Ashton:

- Assistance with experimental design and interpretation of results
- Editing of chapter

Andrei Alexa:

- Cooperative optimisation of transfection parameters using flow cytometry

Selected data from this chapter have been published in the *American Journal of Physiology* - Heart and Circulatory Physiology.

See Hoe LE, Schilling JM, Tarbit E, **Kiessling CJ**, Busija AR, Niesman IR, Du Toit E, Ashton KJ, Roth DM, Headrick JP, Patel HH, Peart JN. Sarcolemmal cholesterol and caveolin-3 dependence of cardiac function, ischemic tolerance, and opioidergic cardioprotection. *Am J Physiol Heart Circ Physiol.* 2014 Sep 15;307(6):H895-903.

5.1 INTRODUCTION

As previously shown in Chapter 3, a decrease in Cav3 expression is associated with the age-related decline in I-R tolerance. Caveolae influence cell signalling by sequestering receptor proteins including GPCRs (and their subunits) and receptor tyrosine kinases (RTKs), these receptor families have all previously been shown to play crucial roles in cardioprotection (as reviewed by Stary et al. 2012 and discussed in detail in Section 1.7.1.3). The Cav3 structural protein is critical for caveolae formation in muscle-type cells including cardiomyocytes as demonstrated through *Cav3*-deficient mice whose hearts lack caveolae (Park et al. 2002). *Cav3*-deficient hearts also display reduced cardioprotection via the loss of IPC, anaesthetic and GPCR-mediated preconditioning, demonstrating the importance of caveolae in cardioprotection (Tsutsumi et al. 2008; Horikawa et al. 2008). Furthermore, pharmacological disruption via MβCD treatment results in depletion of membrane cholesterol, disrupting caveolae structure and reducing recovery from I-R (See Hoe et al. 2014; Horikawa et al. 2008; Patel et al. 2006; Tsutsumi et al. 2010). In further support of our evidence, aged (74-week old mice) mice have been shown to demonstrate a reduction in cardiac caveolae density (Kidd et al. 2010; Peart et al. 2014). Conversely, cardiomyocyte-specific Cav3 overexpression results in the restoration of caveolae density in the aged heart to comparable levels displayed in young wild-type hearts thus leading to improved ischaemic tolerance and recovery in these aged hearts (Kidd et al. 2010). A limitation of *Cav3*-knockout and pharmacological methods is that these result in complete ablation of caveolae. Therefore, we sought an approach to reduce rather than remove caveolae entirely. For this purpose, we investigated the application of RNA interference (RNAi).

RNA interference (RNAi) is an important natural pathway used in many different organisms to regulate gene expression. RNAi typically results in the reduction of gene expression and the corresponding protein product by as much as 70-90%, although the degree of knockdown is highly dependent on cell type (Ma, Lin & Qiu 2012). This pathway was first discovered by Fire, Mello and colleagues in 1998 who published their seminal article describing endogenous post-transcriptional mechanisms of suppressing mRNA expression in *C. elegans* (Fire et al. 1998). For this discovery they were awarded The Nobel Prize in Physiology or Medicine in 2006.

RNAi exploits endogenous mechanisms that use small RNA fragments such as small interfering RNA (siRNA) and microRNA (miRNA) which are used within the cell to fine-tune transcriptional activity. Current estimates suggest there are over 1,000 known miRNA in the mammalian genome (http://microrna.sanger.ac.uk). Experimental & bioinformatics evidence suggests that >30% of protein-coding genes are subject to miRNA-mediated regulation (Chen & Rajewsky 2007). Whilst typically involved in gene suppression some evidence exists demonstrating miRNAs can also result in gene activation (Zamore & Haley 2005).

MicroRNAs are transcribed from their own specific genes or from within introns of protein-coding genes (Saini, Jones & Enright 2007). MicroRNA is first transcribed as a primary miRNA (pri-miRNA) of few hundred nucleotides folded into a hairpin loop structure (Figure 5.1A). The pri-miRNA is then trimmed by the Drosha enzyme in the nucleus to form a pre-miRNA transcript of approximately 70 nucleotides in size (Figure 5.1A). The pre-miRNA is transported out of the nucleus via the Exportin-5 protein. Once in the cytoplasm, the enzyme Dicer removes the hairpin loop, resulting in a mature miRNA duplex approximately 22-23 base pairs in size (Figure 5.1B). One strand (known as the 'guide' strand) of the mature miRNA duplex is captured by a multi-protein complex known as RNA-induced silencing complex (RISC), the accompanying 'passenger' miRNA strand is typically degraded (Figure 5.1C). The RISC complex uses the miRNA-guide strand for mRNA target recognition through base complementarity using the Argonaute family of proteins (Pratt & MacRae 2009). Through Argonaute protein subunits, RISC possesses RNase activity and cleaves the target mRNA if perfect complementarity between the miRNA and target mRNA occurs (Figure 5.1D). Whilst, imperfect base-pairing between the miRNA and target mRNA does not result in cleavage of the target mRNA, gene silencing can still occur through translational repression (Figure 5.1E). As a consequence of mRNA degradation or translational repression *de novo* protein synthesis is reduced with varying efficacy. Alternatively, double-stranded (dsRNA) can be introduced exogenously as small 20-30 nucleotide molecules known as small interfering RNA (siRNA). These siRNA molecules can also result in RNAi using similar pathways to miRNA-mediated silencing. It is thought that miRNAs are responsible for gene regulation whilst siRNAs are defensive mechanisms for genomic integrity in response to foreign or invasive nucleic acids such as viruses, transposons and transgenes (Carthew & Sontheimer 2009).

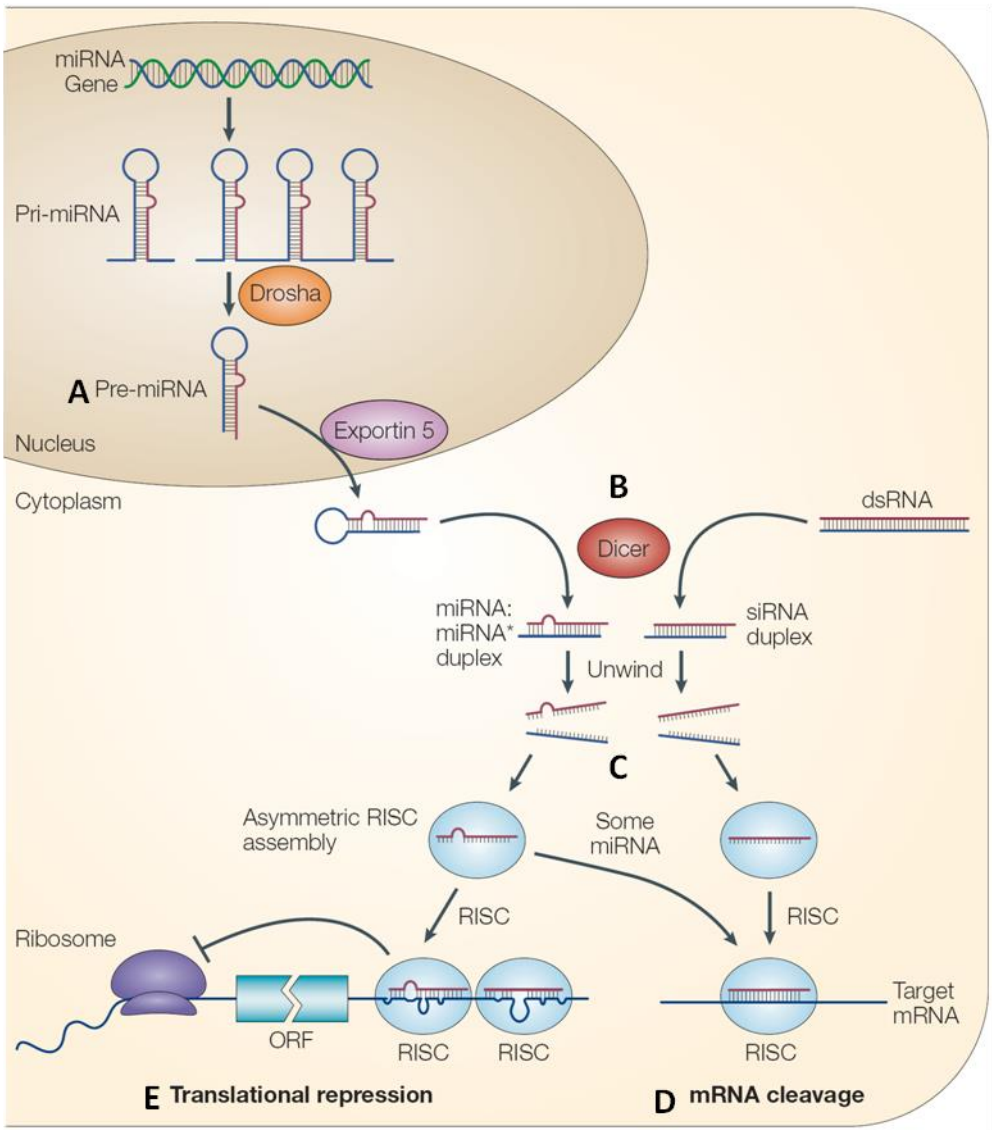


Figure 5.1 The RNAi pathway (Taken from He & Hannon 2004). Experimental introduction of miRNAs and dsRNAs rely on the same endogenous RNAi mechanisms that occurs naturally within the cell.

The natural process of RNAi can be experimentally manipulated by adding exogenous miRNA genes into cells to target and repress specific mRNA sequences. As an experimental tool of investigating gene knockdown there are several advantages to performing RNAi over knockout animals and pharmacological approaches. Unlike knockout-organisms where there is total depletion of target mRNA, RNAi allows for a reduction rather than ablation of the target mRNA and corresponding protein expression. Using RNAi, a reduction of as much as 70-90% knockdown can be achieved (Hsieh et al. 2004). Traditional gene knockout approaches result in an animal that is deficient in the target gene and resulting protein. This can result in embryonic lethality which has been estimated to occur in 15% of knockout models (NIH 2003). In addition, some knockout models may result in compensatory mechanisms occurring. For example, *MyoD* or *Myf5* deficient mice both demonstrate the wild-type phenotype. However, the *MyoD/Myf5* double-knockout shows complete deficiency of both genes and a distinct phenotype (Rudnicki et al. 1992; Rudnicki et al. 1993). When compared to pharmacological approach, RNAi is more readily employed since it relies on mRNA sequence knowledge which can be designed to more specifically target a single mRNA and its resulting protein product. In contrast, many pharmacological approaches are broad action or use indirect actions to achieve a desired effect. For example, MβCD results in caveolae disruption through cholesterol depletion which will affect other cholesterol containing cellular compartments (including planar lipid rafts) and hence may have an undesired effect on cells (Mathay & Poumay 2010). To avoid this, *Cav3* RNAi has been used by others to selectively target caveolae for use in patch-clamp studies to avoid confounding effects of lipid raft depletion (Garg, Jiao & Hu 2009; Balijepalli et al. 2006). RNAi allows for down-regulation of the target protein without completely ablating its expression which may more closely resemble expression perturbations in disease states.

Experimentally, RNAi can be induced by exogenous introducing miRNA expression vectors or siRNA molecules. There are three major technologies used to introduce these exogenous molecules into target cells: 1) transfection using cationic lipids or polymers, 2) electroporation and 3) viral transduction. Transfection is the most commonly used approach in cell culture investigations. This is mainly due to its safety, relative experimental ease and high efficiency and reproducibility in dividing cells. However, this approach has a low efficiency of delivery in primary and non-dividing cells, as well as in *in vivo* models (Karra & Dahm 2010). A further extensive comparison of the different delivery methods is beyond the scope of this study, but is reviewed elsewhere (Louch, Sheehan & Wolska 2011). Chemical transfection employs

cationic lipids or polymers which contain diacylglycerol formulations such as DOPC and DOPE. These combine with DNA to form moieties that cross the plasma membrane and enter into the cell's nucleus through currently unknown mechanisms (Elouahabi & Ruysschaert 2005). Once introduced the effects of transfection are typically transient with the miRNA effects lasting for 2-3 days, as the introduced vector isn't integrated into the genome. As a consequence, the vector is diluted out during cell division or degraded by nucleases.

As previously discussed transfection is most effective in dividing non-primary cells. For this reason, we used the HL-1 cardiomyocyte cell line as the experimental model. The HL-1 cardiomyocyte cell line is an SV40 immortalised cardiac cell line which has been shown to resemble adult cardiomyocyte counterparts by expressing adult marker genes and retains a cardiac morphology even after high passage counts (Claycomb et al. 1998). More specifically, key morphological features such as ANF granules, formation of intercalated discs and sarcolemmal Z densities which have all been shown to be present and retained following serial passages (Claycomb et al. 1998). Despite continual mitosis, HL-1 cells also display adult phenotypic markers such as α-MHC, ANF, α-actin cardiac and Connexin43 (Claycomb et al. 1998). Although they do not express beta-tubulin II which is found in primary atrial cells (Kuznetosv et al. 2015). Similar to its adult primary counterpart, HL-1 cardiomyocytes spontaneously contract as shown by electrophysiological characterisation (Claycomb et al. 1998). HL-1 cells also express many receptors and their respective cytoplasmic targets, this is further demonstrated their ability to be pharmacologically preconditioned model prior to simulated I-R (Seymour et al. 2003). Collectively, HL-1 cardiomyocytes have become an alternative cell-culture model to neonatal and adult cardiomyocyte in the context of simulated I-R (Parameswaran et al. 2013). Furthermore, HL-1 cells do not lose their adult cardiomyocyte phenotype and significant attrition once isolated, whereas adult primary cardiomyocytes have been shown to have reduced T-tubule density and capacitance as early as 24 hours post-isolation (Louch, Sheehan & Wolksa 2011; Zhou et al. 2000). HL-1 cardiomyocytes are also amenable to transfection via cationic lipids, as commonly used in RNAi (Brady et al. 2007; Huang et al. 2010). In the following study we developed protocols to introduce miRNA-*Cav3* expression vectors into HL-1 cardiomyocytes via cationic lipid transfection. A number of parameters were assessed to optimise transfection efficiency to maximise Cav3 knockdown and enable the analysis of the functional effects, specifically changes in ischaemic tolerance.

5.2 METHODS

5.2.1 Cell culture

HL-1 cardiomyocytes were cultured and maintained as described in Section 2.1.2. For all transfection experiment, HL-1 cells were kept in a logarithmic growth phase at 70-80% confluency. We employed HL-1 cells for transfection and sI-R experiments as shown by several authors (Brady, Hamacher-Brady and Gottlieb, 2006; Wang et al. 2013; Brady et al. 2007).

5.2.2 Vector construction and purification

Using the online design tool provided by the manufacturer, four pre-designed BLOCK-iT™ miR RNAi Select oligonucleotide sequences (Mmi505775; Mmi505776; Mmi505777; Mmi505778) targeting different regions of the mouse *Cav3* mRNA (Table 5.1 and Figure 53) were cloned in to pcDNA™6.2-GW/EmGFP-miR BLOCK-iT™ Pol II miR RNAi expression vectors according to supplier's protocols (Life Technologies, Carlsbad, CA, USA). To control for non-specific RNAi effects, an expression vector containing a miRNA targeting the bacterial *LacZ* gene was also constructed for use as a negative control. The miRNA vectors were transformed into TOP10 *E. coli*, propagated for 24 hours, and five individual colonies for each miRNA sequence purified using the PureLink™ HQ Mini Plasmid Purification kit (Life Technologies). Plasmid DNA concentration and purity was assessed via UV spectrophotometry (NanoDrop 2000; Thermo Scientific, Waltham, MA, USA). Plasmid integrity was also assessed by 1% agarose gel electrophoresis (90V; 90 minutes). Finally, to verify successful and accurate insertion of *Cav3* and *LacZ* oligonucleotides into the vector, all miRNA vectors were Sanger sequenced using BigDye Terminators according to the manufacturer's protocols (Life Technologies, Carlsbad, CA, USA). Sequencing chromatograms were analysed using FinchTV software (PerkinElmer Seattle, WA, USA).

Table 5.1 *Cav3* and *LacZ* miRNA duplex sequences used to create artificial miRNA hairpins. The mature miRNA sequence is underlined on the top strand.

Vector ID	BLOCK-iT Identifier	Strand	Sequence (5'-3')
Cav3-A	Mmi505775	Top	TGCTGACAATGTCCTCATTGATGTTCGTTTTGGCCA
			CTGACTGACGAACATCAGAGGACATTG
		Bottom	CCTGACAATGTCCTCTGATGTTCGTCAGTCAGTGGC
			CAAAACGAACATCAATGAGGACATTGTC
Cav3-B	Mmi505776	Top	TGCTGTTCCCTTCGCAGCACCACCTTGTTTTGGCCA
			CTGACTGACAAGGTGGTTGCGAAGGGAA
		Bottom	CCTGTTCCCTTCGCAACCACCTTGTCAGTCAGTGGC
			CAAAACAAGGTGGTGCTGCGAAGGGAAC
Cav3-C	Mmi505777	Top	TGCTGAGATGTGGCTGATGCACTGGAGTTTTGGCCA
			CTGACTGACTCCAGTGCCAGCCACATCT
		Bottom	CCTGAGATGTGGCTGGCACTGGAGTCAGTCAGTGGC
			CAAAACTCCAGTGCATCAGCCACATCTC
Cav3-D	Mmi505778	Top	TGCTGAACAGGCGGTAGCACCAGTACGTTTTGGCCA
			CTGACTGACGTACTGGTTACCGCCTGTT
		Bottom	CCTGAACAGGCGGTAACCAGTACGTCAGTCAGTGGC
			CAAAACGTACTGGTGCTACCGCCTGTTC
LacZ	-	Top	TGCTGAAATCGCTGATTTGTGTAGTCGTTTTGGCCA
			CTGACTGACGACTACACATCAGCGATTT
		Bottom	CCTGAAATCGCTGATGTGTAGTCGTCAGTCAGTGGC
			CAAAACGACTACACAAATCAGCGATTTC

ATGATGACCGAAGAGCACACGGATCTGGAAGCTCGGATCATCAAGGACATTCACTGCAAGGA
GATAGACTTGGTGAACCGCGACCCCAAGAACATCAATGAGGACATTGTGAAGGTAGATTTTG
AAGACGTGATTGCGGAGCCCGAGGGCACCTACAGCTTCGACGGTGTATGGAAGGTGAGCTTC
ACCACGTTCACCGTCTCCAAGTACTGGTGCTACCGCCTGTTGTCTACACTGCTGGGTGTTCC
ACTGGCCCTGCTCTGGGGATTCCTGTTCGCCTGTATCTCCTTCTGCCACATCTGGGCCGTGG
TGCCCTGCATTAAGAGCTACCTGATCGAGATCCAGTGCATCAGCCACATCTACTCACTGTGT
ATCCGCACCTTCTGCAACCCGCTCTTCGCTGCGTTGGGCCAGGTCTGCAGCAACATTAAGGT
GGTGCTGCGAAGGGAAGGCTAA

Figure 5.2 Alignment of the four Block-iT miR-*Cav3* sequences to the coding sequence of *Cav3* mRNA. *Cav3* miRNA target sequences are underlined (*Cav3-A, Cav3-D, Cav3-C* and *Cav3-B* respectively). Exons 1 and 2 are identified by the black and blue text respectively.

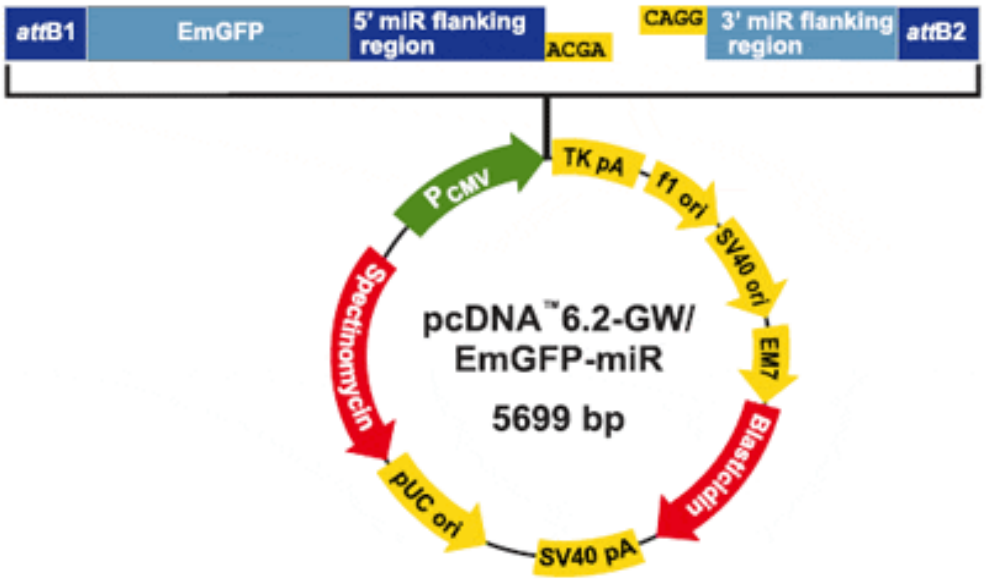


Figure 5.3 Map of the pcDNA™6.2-GW/EmGFP-miR vector. Each miRNA is inserted between the 5' and 3' miR flanking regions. Key features of this plasmid are the CMV promoter (P_{CMV}), spectinomycin resistance gene and the Emerald Green Fluorescent Protein (EmGFP) gene which are co-expressed with the miRNA and allows for visual assessment of transfection efficiency (Lipofectamine 2013).

5.2.3 Transfection

Five commonly used chemical transfection reagents were initially trialled using suggested manufacturers' protocols: FuGene (Promega), Lipofectamine LTX® (Life Technologies), TransFectin® (Bio-Rad), Lipofectamine® 2000 (Life Technologies) and DharmaFECT1® (GE Healthcare). Lipofectamine® 2000 performed the best in the preliminary trials and was used in all subsequent transfections. Further optimisation of transfection protocols was carried out according to manufacturer's protocols (Life Technologies, Carlsbad, CA, USA). Briefly, this involved systematically addressing each of several critical variables including (but not exclusive to) DNA concentration, vector DNA:Lipofectamine® 2000 ratio, cell confluency, transfection method (forward or reverse transfection), and cell incubation time in transfection complex. All transfections were performed in a final volume of 100 µL for Lipofectamine® 2000 in 12-well culture plates (Nunc; Thermo Scientific Waltham, MA, USA). The *LacZ*-miRNA vector was used during transfection optimisation to limit any potential deleterious effects of *Cav3* knockdown. Transfection complexes were formed using 50 µL of Opti-MEM media (Life Technologies) and vector DNA. Algorithmic parameters and more specific details are further provided in the results section. Cells were also transfected with transfection reagent alone as a mock control. Cells were transfected with the miRNA vector for 5 hours then washed twice with PBS before the addition of fully supplemented Claycomb media. Transfection efficiency was assessed via GFP expression 48 hours' post-transfection by both fluorescent microscopy and flow cytometry. Based on the optimisation results the following standard protocol: 1 µg vector DNA; 1 µL Lipofectamine® 2000 and a density of 2 x 10^5 cells were applied when transfecting with the *Cav3*-miR expression vectors.

5.2.4 Assessment of transfection efficiency

Prior to the flow cytometric analysis of GFP expression, transfection efficiency was estimated via an inverted fluorescent microscope (EVOS® fl; Life Technologies) at 10X magnification. Transmitted light channel images were overlayed with the GFP channel to show GFP positive cells within the whole population. Flow cytometric assessment of GFP expression was performed on a BD FACSVerse (BD Sciences, East Rutherford, New Jersey, USA). Post-transfection (48 hours) HL-1 cells were trypsinised and then washed with PBS. Cells were stained with the cell viability dye 7-aminoactinomycin D (7-AAD) (BD Pharmingen East

Rutherford, New Jersey, USA) and incubated for 15 minutes. Using 488 nm lasers, fluorescence for GFP and 7-AAD was collected in the FL1 and FL3 channels respectively. Scatter plots, quadrants and fluorescence compensation were established using single population staining for both fluorescent channels as described by MIT (2015). All flow cytometer results were collected using BD CellQuestPro and FACSuite software (BD Sciences).

5.2.5 Simulated Ischaemia-Reperfusion (sI-R)

Simulated ischaemia-reperfusion (sI-R) of HL-1 cells was carried out according to previously published protocols (Brady, Hamacher-Brady and Gottlieb, 2006). Briefly, non-transfected and transfected HL-1 cardiomyocytes were switched to an ischaemia-mimetic solution (IMS) containing 20 mM HEPES (pH 6.6), 125 mM NaCl, 8 mM KCl, 1.2 mM KH_2PO_4, 1.25 mM $MgSO_4$, 1.2 mM $CaCl_2$, 6.25 mM $NaHCO_3$ and 5 mM Na-lactate in a sealed hypoxic chamber (STEMCELL Technologies, Vancouver, BC, Canada) and equilibrated with 95% N_2/5% CO_2 for 3 hours. All chemicals were purchased from Sigma-Aldrich. Reperfusion was achieved by substitution of ischaemia-mimetic buffer with a normoxic Krebs-Henseleit solution containing 20 mM HEPES (pH 7.4), 110 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.25 mM $MgSO_4$, 1.2 mM $CaCl_2$, 25 mM $NaHCO_3$ and 15 mM glucose, equilibrated with 95% O_2/5% CO_2 for a further 5 hours. For normoxic experiments HL-1 cells were incubated in Krebs-Henseleit solution for 8 hours at 37^oC, 95% air/5% CO_2. LDH activity was measured in supernatants using the Cytotox 96® cytotoxity assay (Promega, Madison, WI, USA). Cells were incubated at room temperature and protected from light for 30 minutes prior to the addition of Stop Buffer. Absorbance at 492 nm was measured using a Modulus II Microplate reader (Promega).

5.2.6 Apoptosis detection

To determine apoptosis after normoxia and sI-R, trypsinised cells from each well were resuspended in 1X of Binding Buffer from the Annexin V-PE kit (BD Pharmingen, East Rutherford, New Jersey, USA). Briefly, HL-1 cells were stained using 5 µL of Annexin V and 5 µL of 7-AAD and incubated for 15 minutes. Flow cytometry was carried out on a BD FACSVerse (BD Biosciences) equipped with an argon laser. A total of 1 x 10^4 events were collected per sample. The data were analysed with FACSuite software (BD Sciences, San Jose, CA).

Adjustment of forward scatter, side scatter and FL channel quadrants were setup using similar methods as outlined in Section 5.2.4.

5.2.7 Treatment with MβCD

The effects of *Cav3* RNAi knockdown were compared to those of complete cholesterol depletion via MβCD treatment. Prior to simulated I-R, HL-1 cells were treated with 1 mM MβCD and incubated for 60 minutes in fully supplemented Claycomb media (10% FBS, 100 µg/ml Penicillin/Streptomycin, 0.1 mM norepinephrine, 2 mM L-glutamine) then washed twice with PBS prior to the addition of IMS solution. Normoxic HL-1 cells incubated with MβCD were analysed for drug toxicity using flow cytometric analysis.

5.2.8. cDNA synthesis, RT-qPCR

To assess *Cav3* mRNA knockdown, total RNA was isolated from 4.0 x 10^4 HL-1 cells and used to synthesise cDNA using the FastLane Cell cDNA kit (Qiagen, Hilden, Germany). Reference gene selection for gene expression was performed as previously described in Section *2.3.2*. RT-qPCR and analysis was carried as described in Section 2.3.1.

5.3 RESULTS AND DISCUSSION

5.3.1 Vector assessment

Following construction, the size and integrity of each miRNA vector was assessed by agarose gel electrophoresis. The properly ligated vector is observed as a 5.8kb band as shown in Figure 5.4. Due to supercoiling the vector migrates quicker through the gel and thus appears smaller in size (Akerman 1998; Cole, Gaigalas & Akerman 2006). Damaged vectors containing DNA nicks can also be identified (Figure 5.4, lowest band in lanes 4 and 12) and were discarded. According to manufacturer's instruction, approximately 20% of all constructed miRNA vectors contain 1-2bp deletions within the miRNA sequence. To assess for this, all miRNA vectors were DNA sequenced using Sanger sequencing (Big Dye Terminators, Invitrogen) to confirm that the correct insert sequence was present (Figure 5.5). Vectors which contained a mutated sequence and nicked DNA were discarded.

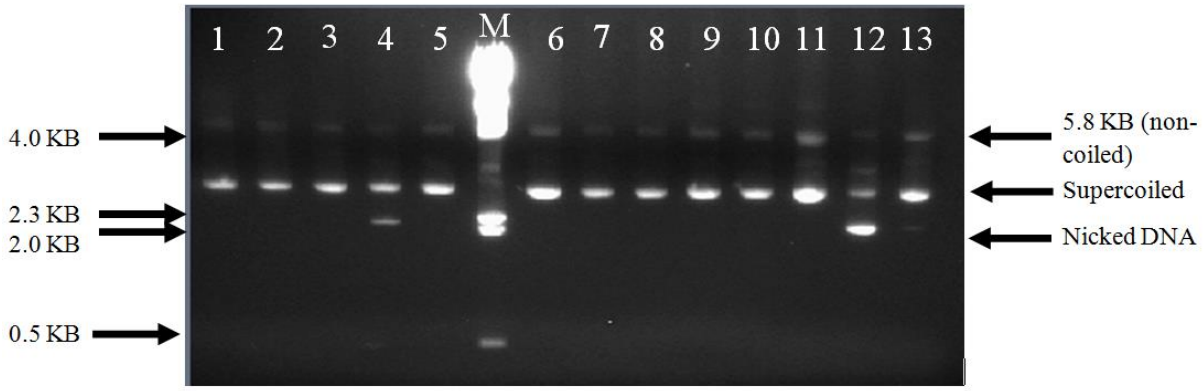


Figure 5.4 Agarose gel analysis of miRNA vectors. Purified miRNA vectors were electrophoresed on a 1% agarose gel at 90V for 90 min. Lane M, Lambda/*Hin*dIII DNA marker; lanes 1-3, miRNA-*Cav3*-A vectors; lanes 4-6, miRNA-*Cav3*-B vectors; lanes 7-8, miRNA-*Cav3*-C; lanes 9-10, miRNA-*Cav3*-D vectors; lanes 11-13, miRNA-*LacZ* vectors.

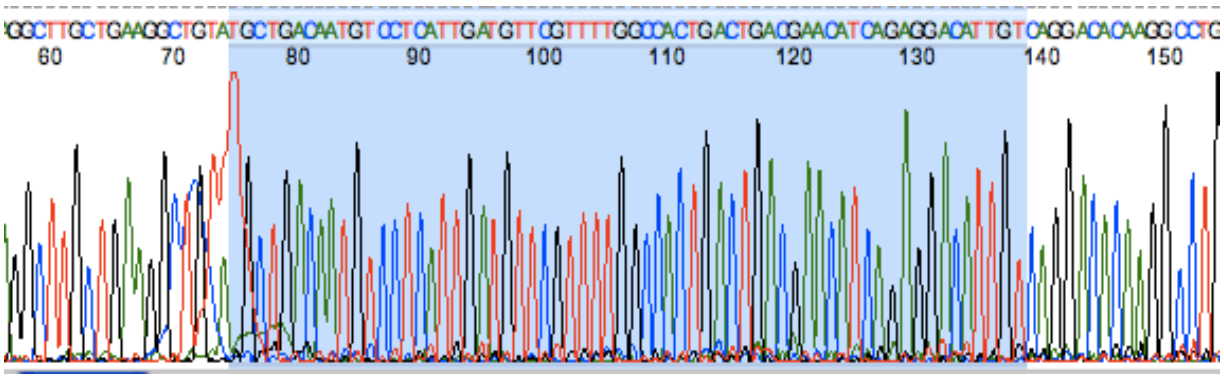


Figure 5.5 DNA sequence confirmation of a miRNA-Cav3 vector. The blue highlighted region corresponds to the miRNA-*Cav3*-A insert sequence flanked by the 5' and 3' miRNA vector regions.

5.*3.2 Assessment of transfection efficiency*

Transfection efficiencies were assessed based on the co-expression of the EmGFP reporter gene in transfected cells. Firstly, cells were observed via fluorescent microscopy to assess for positive transfection and estimate the level of transfection (Figure 5.6). Once confirmed a more accurate assessment of transfection efficiency and cell viability was performed using flow cytometry. Based on flow cytometric data, initial screening of the transfected HL-1 cells demonstrated a small population (20%) of EmGFP positive cells (lower right quadrant) with minimal cell death (upper quadrants) prior to optimisation of transfection parameters (Figure 5.7).

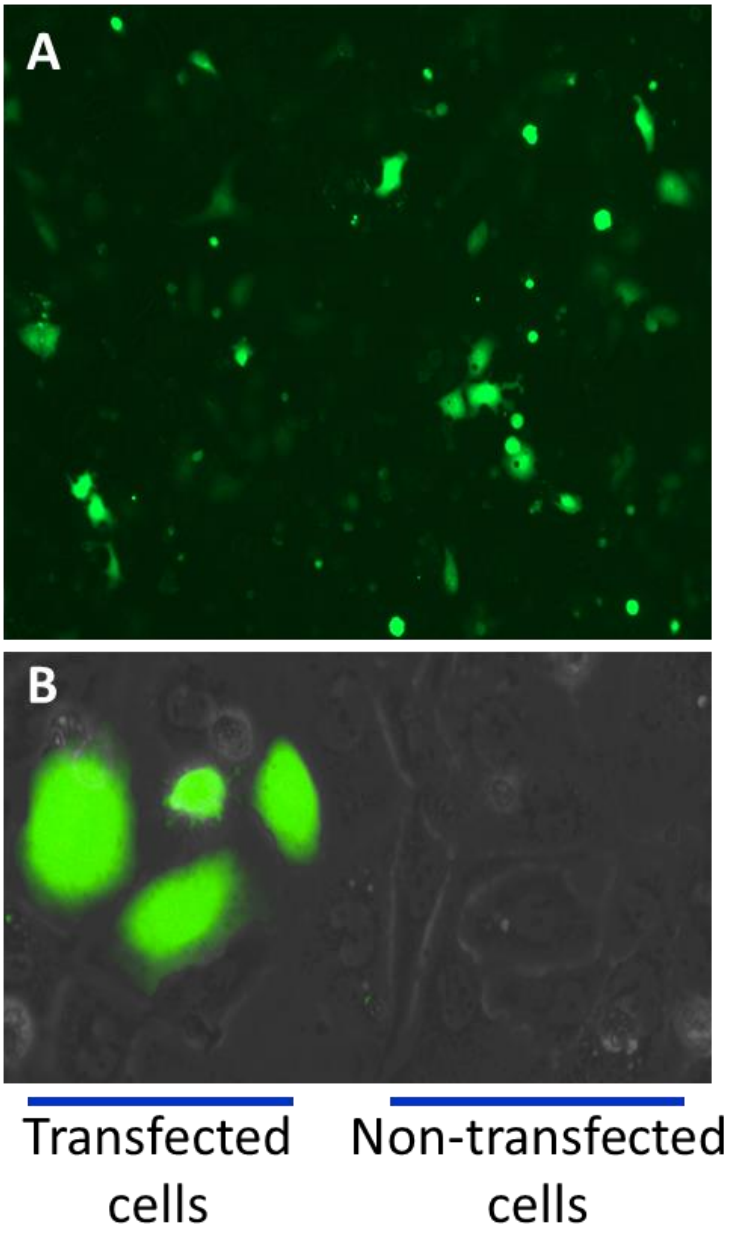


Figure 5.6 Prior to flow cytometric analysis, inverted fluorescent microscopic estimation of EmGFP expression from transfected HL-1 cells was utilised **A)** 40X magnificent **B)** 100X magnification.

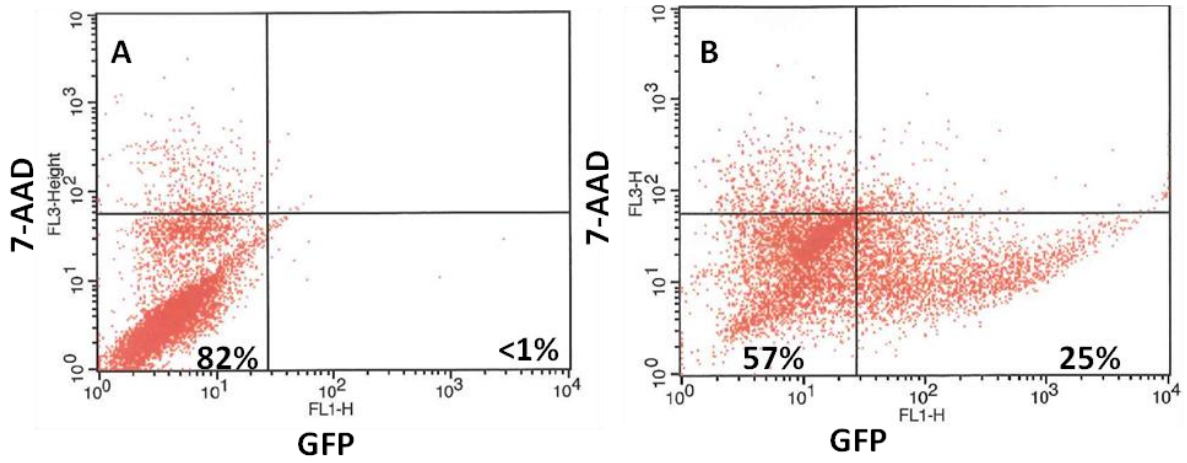


Figure 5.7 Flow cytometric analyses of transfected HL-1 cells. A) Mock-transfected HL-1 cells; **B)** HL-1 cells transfected with pcDNA™6.2-GW/EmGFP-miR-*LacZ* vector. Quadrants correspond to the following phenotypes: Lower left, viable non-transfected; Lower right, viable transfected; upper left, non-viable non-transfected; Upper right, non-viable transfected. The percentage of cells conferring each phenotype is indicated in its respective quadrant.

5.3.3 Transfection optimisation

5.3.3.1 Comparison of popular Liposomal Transfection Reagents

Five commonly used liposomal transfection reagents were initially trialled for suitability using the general parameters supplied by the manufacturer. As shown in Figure 5.8 all five transfection reagents were able to deliver the miR-vector into HL-1 cells, although at varying efficiencies. Using the supplied general experimental parameters Lipofectamine® 2000 demonstrated the highest degree of transfection (40% EmGFP-positive viable cells) with a low level of cytotoxity (<5% EmGFP-positive non-viable cells). This finding matches previous studies which utilised Lipofectamine® 2000 for transfection in HL-1 cardiac cell line (Wang et al. 2013; Brady et al. 2007; Hamacher-Brady, Brady & Gottlieb, 2006). Further refining of the Lipofectamine® 2000 experimental protocol was necessary to maximise transfection efficiency and subsequent gene knockdown. Notable parameters reported to influence transfection efficiency are DNA vector concentration, vector-to-lipid ratio, cell density, and seeding protocol (Dalby et al. 2004).

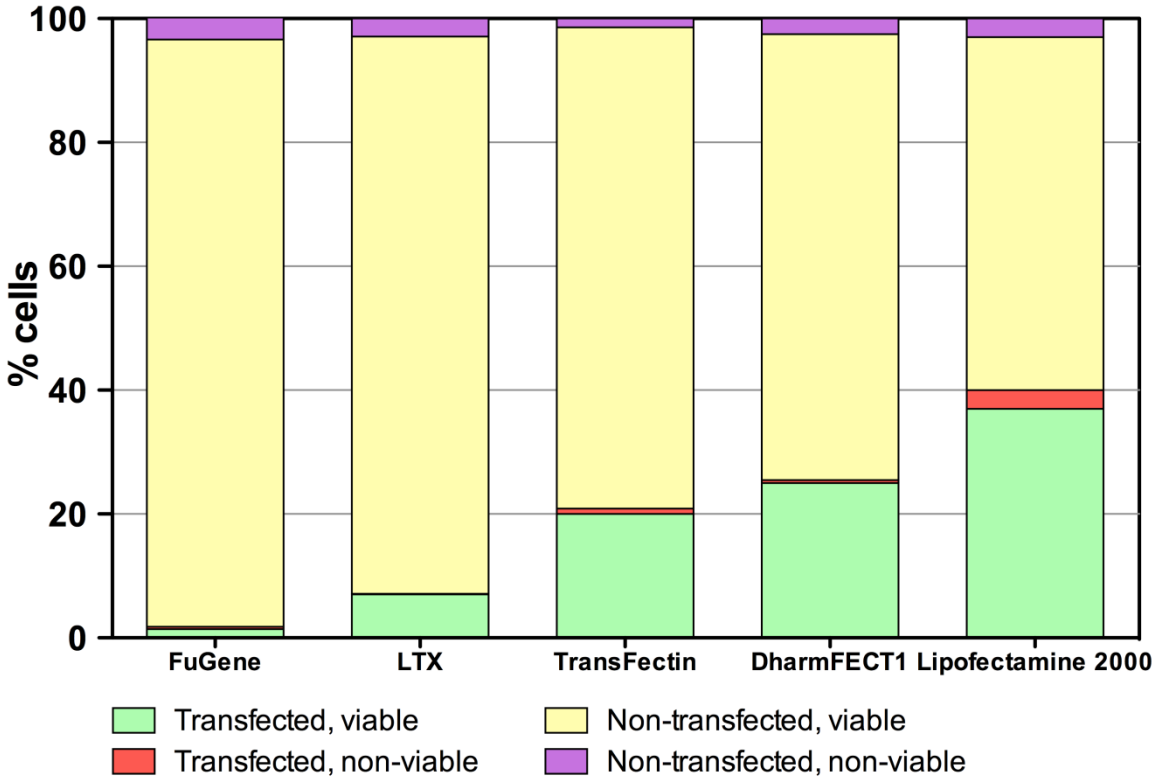


Figure 5.8 Comparison of five transfection reagents. HL-1 cells were transfected with EmGFP-miR-LacZ using different transfection reagents. Flow cytometry was used to assess for transfection (EmGFP expression) and cell viability (7-AAD staining). The percentage of cells conferring each phenotype is indicated in each stacked column.

5.3.3.2 Effect of DNA:transfection reagent ratio

Cationic lipids including Lipofectamine® 2000 are used to transfer nucleic acids into cultured cells as they strongly associate with the negative charge of the DNA phosphate backbone (Ahmad et al. 2005). By modifying the ratio of DNA to cationic lipid ratio an excess of positive charge can be achieved which allows for the complex to closely associate with the negatively charged cell membrane and is thought to assist the DNA-lipid moiety in crossing the plasma membrane (Ewert et al. 2010). This charge ratio can therefore influence the degree and success of transfection, although varying between different cell types. For example, the COS-7 fibroblast cell line has been shown to be more amenable to transfection using lower lipid reagent concentrations compared to the CV-1 fibroblast line which requires higher concentrations of lipid reagent to produce comparable transfection levels (Felgner et al. 1987).

Using 1 µg of vector DNA, titration of Lipofectamine® 2000 reagent was performed in the ranges of 0.5 µL-5 µL as suggested by the manufacturer. Ratios of 1:1 and 1:2 both resulted in over 50% transfection efficiency (Figure 5.9 and Figure 5.10). At higher ratios cytoxicity was observed by an increase in the number of non-viable cells present. Similar cytotoxic effects using high DNA:lipid concentrations have been noted by others (Hofland, Shephard & Sullivan 1996; Felgner et al. 1987).

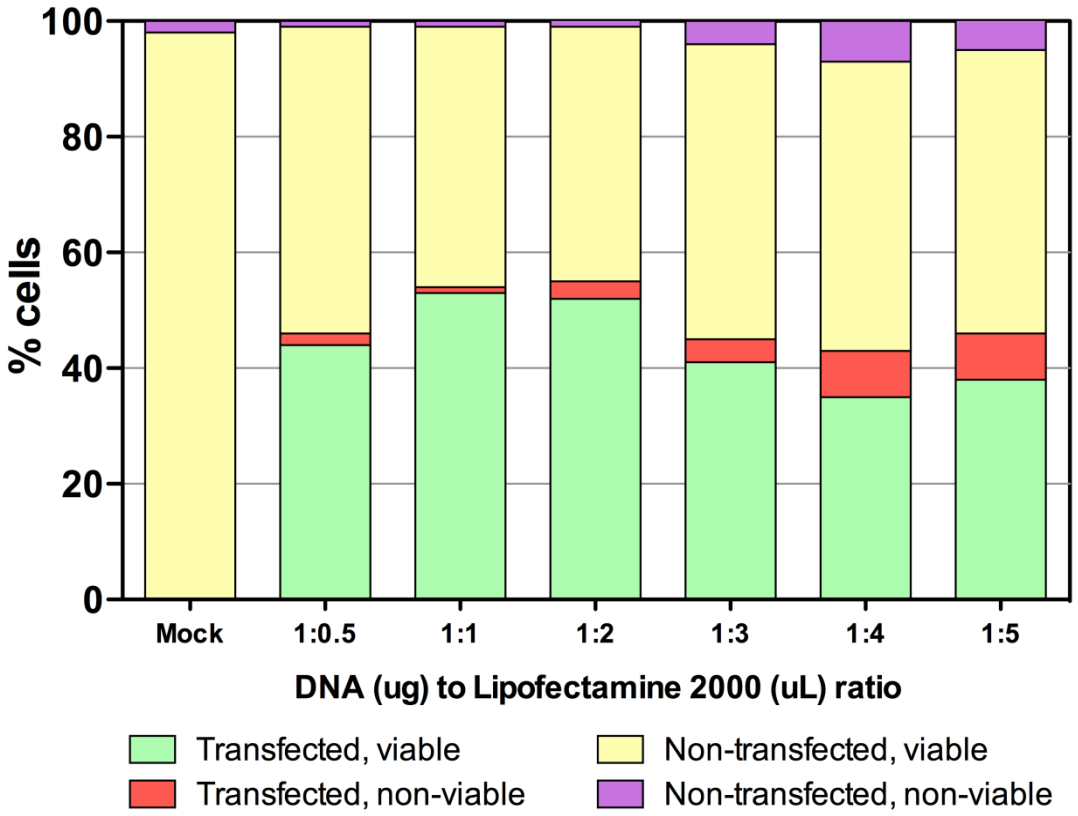


Figure 5.9 Effect of DNA:Lipofectamine® 2000 ratio on transfection efficiency. HL-1 cells (2 x 10^5 cells/well) in 12-well plates were transfected with 1 µg DNA vector and varying concentrations of Lipofectamine® 2000. EmGFP expression and cell viability were measured by flow cytometry. The percentage of cells conferring each phenotype is indicated in each stacked column.

5.3.3.3 Effect of vector DNA concentration

DNA vector concentrations also require optimisation to promote maximal uptake and expression in cells. A range of DNA concentrations (1-4 µg) were examined, with the 1:1 DNA:Lipofectamine® 2000 ratio maintained. Using both 1 µg and 2 µg of DNA vector identical transfection efficiencies (53%) were observed (Figure 5.10). Interestingly the transfection efficiency did not double with a doubling of the DNA:lipid complex concentration. In fact, at higher concentrations (3:3 and 4:4), decreased transfection efficiency and increased cell death was observed. This presumably was due to the cytotoxicity of the higher Lipofectamine® 2000 concentrations as observed previously in Figure 5.9. This data established an upper limit (2 µg) of DNA concentration that could be used without compromising cell viability and transfection efficiency.

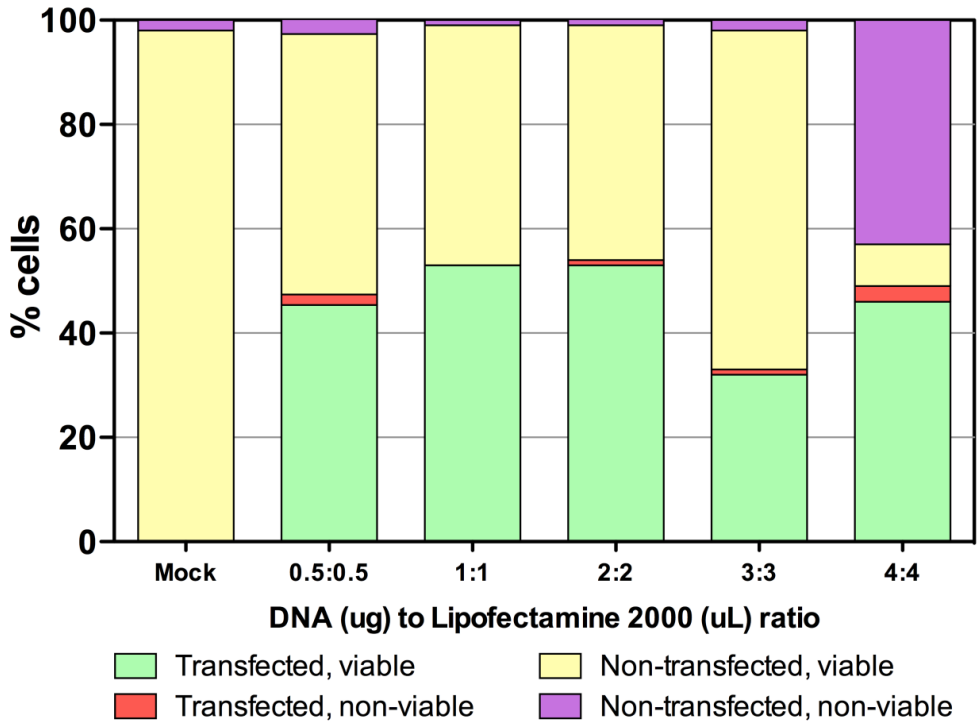


Figure 5.10 Effects of DNA concentration on transfection efficiency. HL-1 cells (2 x 10^5 cells/well) were grown in 12-well plates then transfected with varying concentrations of miRNA-*LacZ* vector and Lipofectamine® 2000 at a fixed 1:1 ratio. GFP expression and cell viability were measured by flow cytometry. The percentage of cells conferring each phenotype is indicated in each stacked column.

5.3.3.4 Effect of cell density

Using 1 µg of vector DNA and a 1:1 ratio, HL-1 cells were assessed for the effects of various seeding densities on transfection efficiency. As observed in Figure 5.11 a seeding cell density of 2.0 x 10^5 cells/well (approximately 70% confluency) gave the highest level of transfection. With lower and higher cell densities showing decreased transfection levels. It is thought that reduced transfection efficiency associated with highly confluent cultures is resultant from inhibition of cell growth and metabolism. Conversely, lower densities can also result in reduced transfection efficiency due to stunted growth with recovery of cells being also affected following transfection (Dalby et al. 2004). Cells should be kept at <80% confluency so they are still in the logarithmic stage of growth, as cationic lipid transfection and reporter expression are increased in mitotically-active cells (Mortimer et al. 1999).

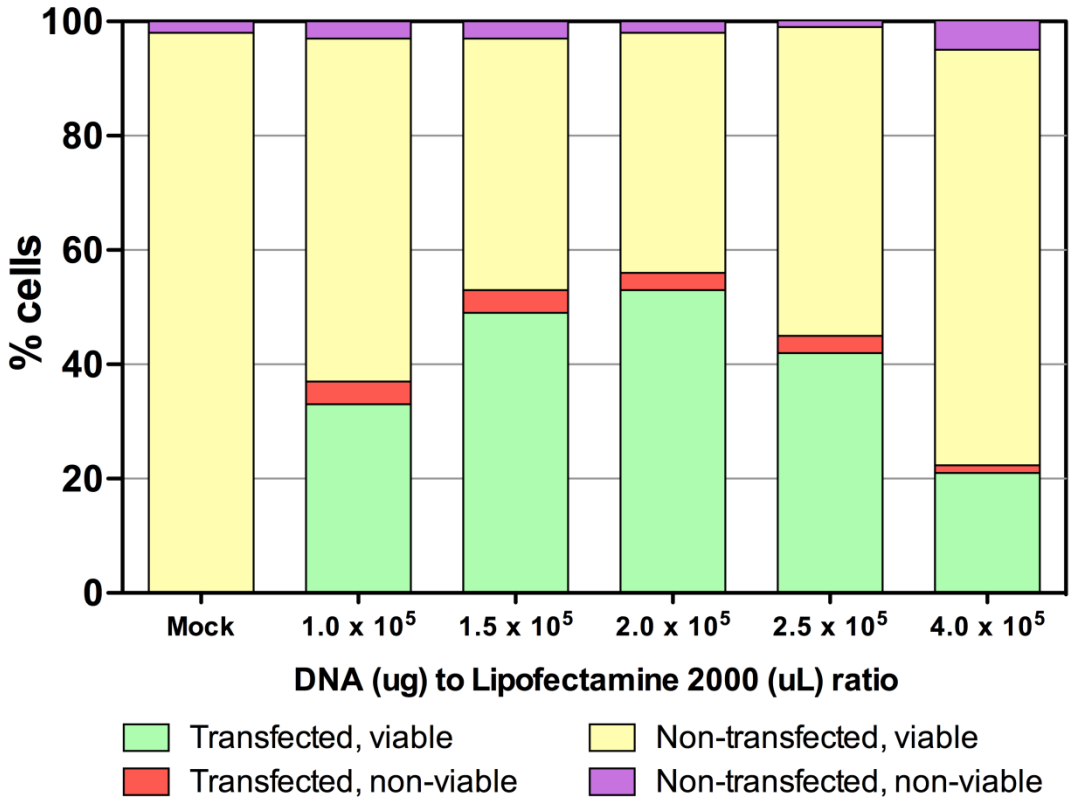


Figure 5.11 Effects of cell density on transfection efficiency. HL-1 cells of different densities (1.0 x 10^5 to 4.0 x 10^5 cells/well) were grown in 12-well plates were transfected with 1 µg of miR-*LacZ* vector and 1 µL of Lipofectamine 2000. GFP expression and cell viability were measured by flow cytometry. The percentage of cells conferring each phenotype is indicated in each stacked column.

5.3.3.5 Effect of reverse transfection

Standard (forward) transfection protocols require cells to be pre-plated into wells and allowed to attach and recover for at least 4-6 hours prior to the addition of the DNA transfection complex. An alternative method known as reverse transfection involves simultaneously transfecting and plating cells. Reverse transfection has been suggested to improve transfection efficiency in some cell lines and reduces handling of the cells when compared to forward attachment protocol (Reid et al. 2009). It has been suggested the improved transfection efficiency using reverse attachment protocol observed in some cell line may be due to trypsinisation which may result in increased surface availability for complexes to enter (Amarzguioui 2004). Reverse transfection of HL-1 cells did not improve transfection efficiency in HL-1 cells as shown in Figure 5.12.

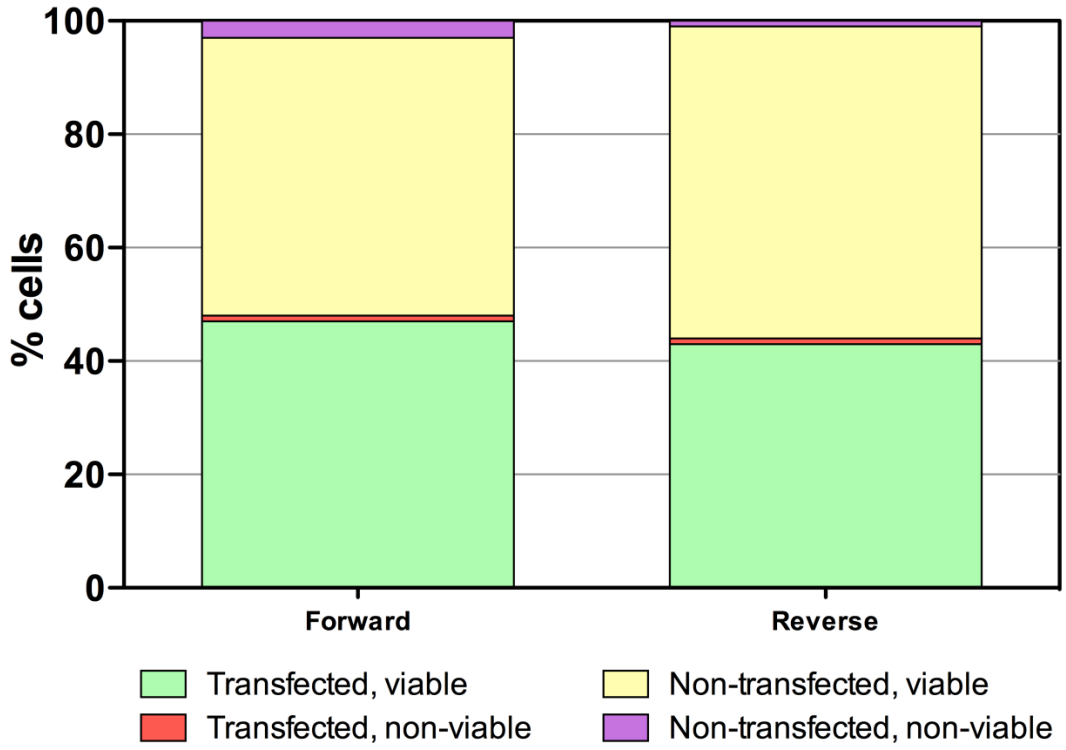


Figure 5.12 Effect of reverse transfection on transfection efficiency. HL-1 cells (2.0 x 10^5 cells/well) grown in 12-well plates than were either pre-plated and allowed to attach for 4-6 hours (forward) prior to transfection (1 µg: 1 µL ratio) or transfected whilst simultaneously plating (reverse). GFP expression and cell viability were measured by flow cytometry. The percentage of cells conferring each phenotype is indicated in each stacked column.

5.3.4 Assessment of Cav3 knockdown

Following optimisation, a transfection efficiency of approximately 40% was obtainable and reproducible. This was comparable to previously reported studies (Wang et al. 2013; Brady et al. 2007; Hamacher-Brady, Brady & Gottlieb 2006). Using this established transfection protocol, HL-1 cells were transfected with each of the four miR-*Cav3* BLOCK-iT vectors. The miR-*lacZ* vector was used as a negative control to control for non-specific transfection effects.

5.3.4.1 Transfection efficiencies in HL-1 cells transfected with miRNA-Cav3 vectors

RNAi using the miRNA vector approach was used to selectively knockdown *Cav3* mRNA, which is responsible for caveolae formation in cardiomyocytes. We chose the miRNA vector approach over small-interfering RNA pathway as the half-life of Cav3 protein was shown to be greater than five hours (for >99% *de novo* protein decay, ≥40 hours is required) (Galbiati et al. 1999). Chemical transfection using Lipofectamine® 2000 reagent was used to introduce miRNA-vectors into HL-1 cardiomyocytes, similar to others (Brady et al. 2007; Hamacher-Brady et al. 2006). As shown in Figure 5.13 all four Cav3-miRNA vectors demonstrated a transfection efficiency of approximately 40%, with the Cav3-A vector demonstrating the highest level (51%) of EmGFP expression.

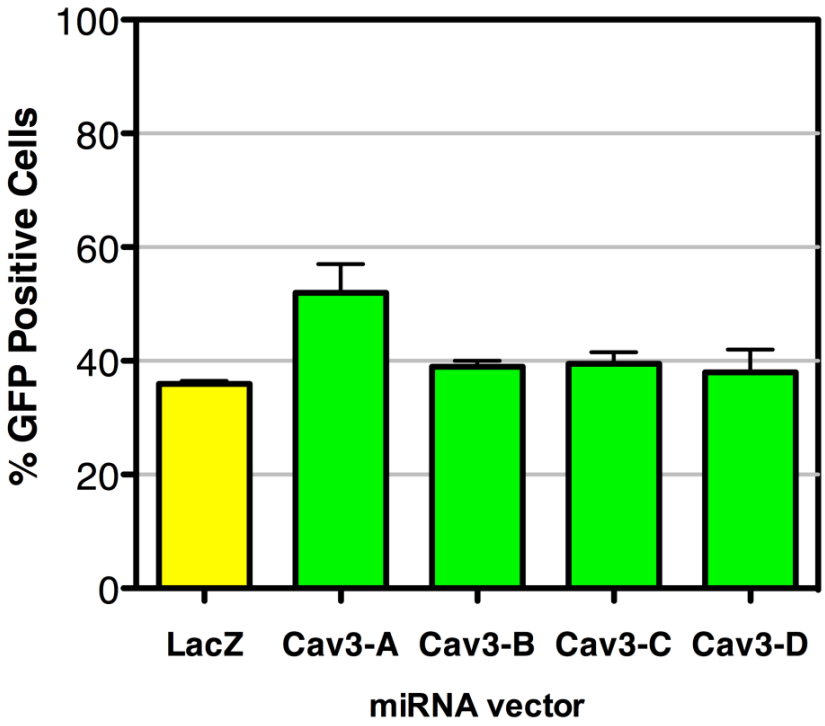


Figure 5.13 Bar graph demonstrating EmGFP expression in transfected cells. Transfection efficiency assessed by flow cytometry in HL-1 cells transfected with each of the four miR-*Cav3* vectors (Cav3-A to Cav3-D) and miR-*LacZ* control vector (n=2/group). Data presented as mean ± SEM.

5.3.4.2 RT-qPCR assessment of Cav3 mRNA knockdown

As previously discussed in Chapter 2.3.2 the choice of a stable reference gene is critical for accurate gene expression analysis. Therefore, a panel of commonly used reference genes (*Actb, Gapdh, Hprt1, Pgk1* and *Ppia*) were examined to determine the most stably expressed gene. As shown in Figure 5.14 *Hprt1* and *Ppia* were the most stably expressed, *Ppia* was selected as the most stable reference gene and was used to normalise gene expression in the miRNA vector transfected cells.

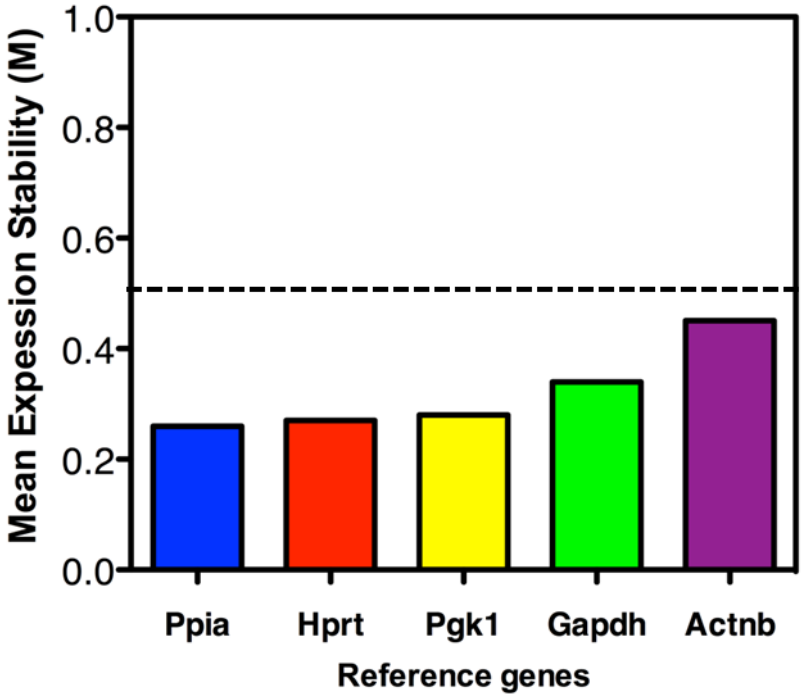


Figure 5.14 Expression stability values (M) of the putative reference genes tested in transfected HL-1 cells. The expression of five genes was analysed to determine the most suitable reference gene. Genes are ranked from the most stable to the least stable (left to right). Dotted line indicates the threshold for stable expression (M-value of <0.5) within homogeneous samples. (n=3/group).

Following 48 hours post-transfection of Cav3 miRNA vectors, the expression of *Cav3* mRNA was assessed relative to the *LacZ* negative control (Figure 5.14). This indicates that the miRNA-*Cav3* expression vectors were expressing miRNA targeting *Cav3* mRNA, resulting in its reduced expression. However, the level of knockdown was considerably less than the expected 70-90% knockdown known to be achievable by RNAi (Sahoo et al. 2009; Schlesinger et al. 2011). The miR-*Cav3*-A vector elicited the greatest degree (33%) of *Cav3* mRNA knockdown. However as only approximately 40% of HL-1 cardiomyocytes were transfected (Figure 5.13), theoretically 60% of HL-1 cells are still expressing normal levels of *Cav3* mRNA. Therefore, a maximal knockdown (>70% repression in transfected cells) would result in a general repression of 28-40% *Cav3* transcript. It has been suggested that transfection efficiencies should be consistently 80% or greater in order to achieve significant mRNA knockdown for RNAi experiments (Wang et al. 2015). For example, transfection efficiencies of <60% have been shown to result in implicated knockdown of 70% observed knockdown, however such an estimate is in doubt as it can barely distinguish itself from a 1% knockdown (Wang et al. 2015). Despite this, statistically significant knockdown was observed with the *Cav3*-A (44%), *Cav3*-C (17%) and *Cav3*-D (17%) miRNA vectors (Figure 5.14). Therefore, further assessment of the Cav3 protein level or the use of the cells in sI-R was abandoned.

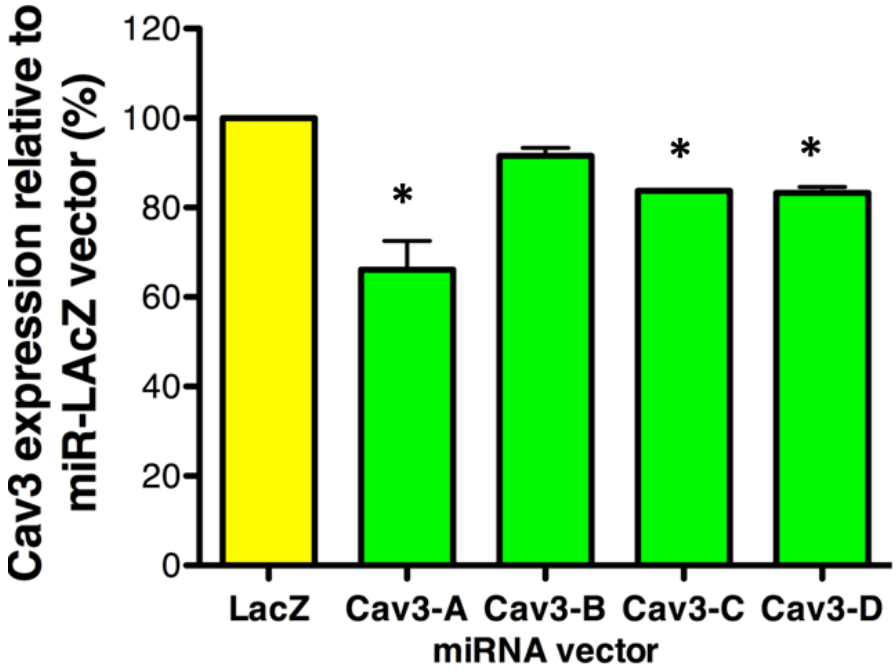


Figure 5.15 Gene expression analysis of *Cav3* mRNA knockdown in miR-Cav3 transfected HL-1 cardiomyocytes. Following transfection with one of the four miRNA-*Cav3* vectors (48 hours) Cav3 mRNA was assessed in HL-1 cardiomyocytes via RT-qPCR (n=3/group). Data presented as mean ± SEM; *P <0.05 relative to miR-*LacZ* negative control.

5.3.5 Cholesterol depletion by MβCD in HL-1 cells

Whilst we were unable to produce a high degree of *Cav3* knockdown in HL-1 cells using transient transfection (44%), pharmacological approach using cholesterol depletion (via MβCD) of cellular rafts were investigated. A concentration of ≥200 μM MβCD has been shown to result in complete loss of caveolae and lipid rafts in cardiomyocytes (Hoe et al. 2014). Firstly, normoxic time-matched HL-1 cells were screened for potential cytotoxic effects of MβCD (1 mM) and was found to be non-cytotoxic as shown by flow cytometric analysis (Figure 5.15A).

Simulated I-R resulted in 17.3% cell death as assessed by flow cytometry (Figure 5.16A) and enhanced LDH release (Figure 5.16B) compared to normoxic controls. As shown by flow cytometry and LDH release, MβCD treatment in sI-R cells resulted in significantly increased total cell death when compared to the sI-R only group (Figure 5.16A-B). Thus suggesting that depletion of caveolae and/or lipid rafts results in a reduction of ischaemic tolerance.

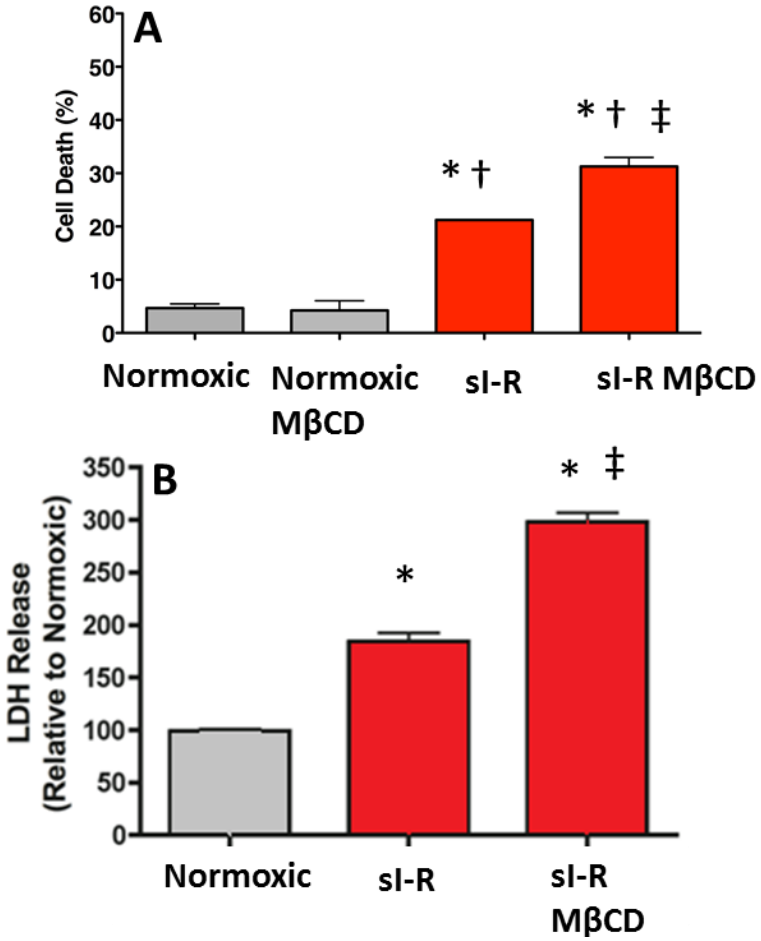


Figure 5.16 Measurements of cell death following sI-R. A) Flow cytometric analysis of total cell death (Annexin-V PE and 7-AAD positive) **B)** LDH efflux analysis of cell viability following sI-R with or without MβCD treatment. Data presented as mean ± SEM; *****, P <0.05, *vs.* normoxic; †, P <0.05, *vs.* normoxic + MβCD; ‡, P <0.05, *vs.* sI-R .(n=3/group).

Prior to sI-R, HL-1 cells were subjected to 60 minutes of MβCD incubation. MβCD treatment (1 mM) has been shown to lower membrane cholesterol and flatten caveolae (Patel et al. 2006). In contrast, others have suggested that MβCD can be cytotoxic (Hinzey et al. 2012). However, our analysis demonstrated that the concentration of MβCD used in this study did not show any cytotoxic effects (Figure 5.16A). MβCD treatment resulted in a reduction of ischaemic tolerance following sI-R, as shown by significantly increased cell death (Figure 5.16A) and LDH release (Figure 5.16B). Flow cytometry and LDH results show similar magnitudes of cell death; with MβCD treatment resulting in ≈50% more cell death when compared to sI-R alone. The decline in ischaemic tolerance may be due to the reduced

autacoid activation of cell membrane receptors (including GPCRs) leading to attenuated survival kinase activation, membrane repair and mitochondrial viability. To support this, Cav3-overexpression in cardiac mitochondria has been shown to reduce calcium loading, enhance respiratory function which results in enhanced preservation of mitochondria associated with increased recovery in these hearts (Fridolfsson et al. 2012). Cav3-overexpression in the heart has been shown to mimic IPC by phosphorylating survival kinases such as Akt and GSK-3β (Tsutsumi et al. 2008). Whilst Cav3 has been shown to be required for sarcolemmal repair as *Cav3*-knockdown results following membrane injury results in reduced plasma membrane resealing leading necrosis in these cells (Corrotte et al. 2013). Indeed, the preservation of sarcolemmal and mitochondrial integrity has been a noticeable feature of Cav3-overexpression (Tsutsumi et al. 2008).

Besides being involved in activation of cardioprotective signalling, caveolae have also been shown to house many ion channels related to the contractile function in many species and models including pig coronary arteries, rat bladder and rat skeletal muscle tissue (Graziani et al. 2004; Cristofaro et al. 2007; Vega Moreno et al. 2012). To highlight its role in contractile function, MβCD treatment of normoxic Langendorff hearts have been shown to lead to significant reduction of LVDP and EDP (Hoe et al. 2014). Similarly, post-ischaemic hearts showed severely reduced recovery following 1 mM MβCD treatment which resulted in approximately 10% recovery of LVDP compared to baseline (Hoe et al. 2014). Conversely, *Cav3* transgenic overexpression restores caveolae formation in the senescent heart to levels observed in the young heart resulting in enhanced post-ischaemic recovery in the senescent hearts (Kidd et al. 2010). Thus methods leading to preservation of caveolae in the aged hearts may be attractive targets for IHD by reducing I-R injury as well as contractile dysfunction.

As previously discussed the success and degree of transfection and resulting knockdown is highly dependent on cell type (Kim & Eberwine 2010). Our extensive optimisation as well as other groups report 40% transfection efficiency in HL-1 cells, thus leaving 60% untransfected population which may present significant hurdles in terms of detecting functional changes. Perhaps to overcome this hurdle, other groups employing HL-1s have selectively studied only GFP positive populations by the use of fluorescence microscopy in the context of autophagy (Brady et al. 2007; Rikka et al. 2011). Using fluorescent microscopy transfected populations can be individually identified and these populations can be surveyed for changes in functional

responses. Terminally differentiated cells such as isolated and cultured cardiomyocytes can be difficult to chemically transfect cells and may require non-liposomal methods to achieve higher efficiencies of gene knockdown (Thiel & Nix 2006). The use of electroporation has been shown to achieve >70% knockdown of target gene in cardiomyocytes, whilst adeno-associated virus (AAV) transduction has been shown to achieve 90% transfection efficiency in adult cardiomyocytes (Louch, Sheehan & Wolska 2011; Dandapat et al. 2008). Indeed, AAV vectors have been used to overexpress Cav3 in cardiomyocytes (Tsutsumi et al. 2008; Rikka et al. 2011) and in the study of autophagy in adult cardiomyocytes (Quinsay et al. 2010). Alternatively, GFP positive cells can be further enriched using a fluorescently activated cell sorter or via stable transfection. Fluorescently activated cell sorters can enrich up to 4.5 x 10^6 GFP positive cells, yielding a homogenous population (>95% GFP), which can then be used for further downstream experiments such as sI-R (Basu et al. 2010). Whilst stable transfection relies on the incorporation of the plasmid DNA into the host genome which is a rare event (>1 in 10^4 cells) and requires the use of cytotoxic drugs for selection (Mortensen et al. 2004). The use of siRNA transfection in HL-1 cardiomyocyte instead of miRNA expression vectors may also improve transfection efficiency (Takeuchi, Kim & Matsuoka 2013; Rikka et al. 2011). It is possible that siRNAs molecules are able to cross the membrane more efficiently than the larger plasmid moieties resulting in higher transfection efficiency and knockdown as seen when combined with electroporation (Louch, Sheehan & Wolska 2011). However, other groups have also noted low transfection efficiencies in cardiomyocytes using liposome-mediated siRNA transfections, which led to the use of AAV vectors as a delivery method (El-Armouche et al. 2007).

5.4 CONCLUSION

In this study, we sought to selectively deplete caveolae formation in HL-1 cardiomyocytes via knockdown of *Cav3* using RNAi. A commonly used cationic lipid, Lipofectamine® 2000 has been shown to significantly increase transfection in primary neurons and amenable to transfection in HL-1 cardiomyocyte cells (Dalby et al. 2004; Wang et al. 2013; Brady et al. 2007). Through extensive optimisation of several crucial transfection parameters, we were able to achieve >40% transfection efficiency in HL-1 cardiomyocytes. This level of transfection efficiency is comparable to previously published studies (Brady et al. 2007; Rikka et al. 2011). Knockdown of *Cav3* at the mRNA level was observed although the degree of reduction was low (reduction of 28-40%). This can be partially attributed to the high number of non-transfected HL-1 cardiomyocytes (≈60%) in the total cell population which still express *Cav3* mRNA at normal levels. As previously discussed even with 100% knockdown in all transfected cells a maximal global reduction of ≥90% is possible. Studies looking at cell-specific ultrastructural changes such as Brady et al. (2007) are not as reliant on high levels of transfection compared to studies looking at global changes (e.g. LDH release, mRNA and protein expression) following gene knockdown. Indeed, Rikka et al. (2011) have used both liposomal and adenoviral approaches to transfect HL-1 cells, perhaps to overcome the challenging chemical transfection efficiency. For these reasons we decided not to further pursue RNAi in HL-1 cells with regards to Western immunoblotting and sI-R.

To support caveolar involvement, we employed non-specific pharmacological depletion of all lipid rafts using MβCD which has been previously shown to ablate caveolae formation in the heart (Horikawa et al. 2008). Loss of GPCR and anaesthetic-mediated loss of cardioprotection following MβCD treatment has been observed (Patel et al. 2006; Horikawa et al. 2008), here we show that MβCD treatment prior to sI-R results in significant attenuation of ischaemic tolerance in cultured cardiomyocytes (Figure 5.15A and 5.15B). However other groups have shown that there are no differences in cell death between MβCD-treated cells and sI-R only group (Horikawa et al. 2008; Tsutsumi et al. 2014). Interestingly MβCD treated sI-R cardiomyocytes show similar expression of apoptosis-related genes (*Casp3*, *BID*, and *BAD*) and anti-apoptotic genes (*Bcl2* and *Iap2*) when compared to sI-R group without MβCD treatment (Tsutsumi et al. 2014). The reasons for these discrepancies are unknown. Notable differences between the studies include the cell culture model, where adult primary

cardiomyocytes were used in place of immortilised HL-1 cardiomyocytes, and sI/R times. Future studies should also examine the expression of these genes in the model used here. Although we were unable to achieve significant knockdown of Cav3 by RNAi, we were able to show caveolar involvement in modifying ischaemic tolerance in HL-1 cardiomyocytes by using cholesterol depletion. In conclusion, caveolae may modify ischaemic tolerance by its involvement in signal transduction from the membrane leading to activation of survival kinases, plasma membrane repair mechanisms and mitochondrial protein-S nitroyslation.

6. Chapter Six

Expression of miRNAs associated with ischaemia, senescence and caveolae transcripts in the ageing heart.

ADDENDUM

Contributions to Chapter 6:

Can Kiessling:

- Experimental design
- All laboratory bench-work
- Data analysis
- Author of the chapter

Kevin Ashton:

- Assistance with experimental design and interpretation of results

Selected data from this chapter have been presented at

Kiessling C.J., Reichelt M.E., Headrick J.P., Ashton K.J. 2015. Candidate MicroRNA Expression May Contribute to Post-Ischaemic Transcriptional Changes in Ageing Mouse Hearts. Gold Coast Health and Medical Research, Gold Coast.

6.1 INTRODUCTION

As discussed in the preceding chapter, miRNAs are a class of small (typically 18-25 nucleotides in size) single-stranded, non-coding RNAs that regulate gene expression at the post-transcriptional level. Their regulatory roles have been implicated in most biological processes including cell proliferation, development, differentiation and apoptosis (Kuppusamy, Sperber & Ruohala-Baker 2013). Based on recent estimates, the human and mouse genome contains >1,000 known miRNAs which are thought to regulate >30% of the genome (http://microrna.sanger.ac.uk) with the mouse heart shown to express over 300 miRNAs as determined by high throughput sequencing (Matkovich 2014). As a single miRNA can target more than one mRNA, miRNAs can regulate multiple downstream targets affecting multiple regulatory networks in the cell. For example, *miR-1* has been implicated in targeting multiple components of MAPK (i.e. p38 and phosphor-Erk1/2) signalling as well as targeting the sodium-calcium exchanger *Ncx1* in the heart (Karakikes et al. 2013; Kumarswamy et al. 2012). Differential expression of miRNAs has been associated with a variety of diseases including cancer, autoimmune and cardiovascular disease (Li & Kowdley 2012). More specifically, the involvement of miRNAs has been observed in cardiovascular diseases such as myocardial infarction, heart failure, and cardiac hypertrophy. In addition, dysregulation of miRNA has been associated with ageing and senescence in the heart [Jung & Suh 2012; Ucar et al. 2012].

As previously discussed, the ageing heart has been shown to involve complex molecular and cellular changes. These include increased fibrosis with a loss of contractile tissue, mitochondrial DNA deletions and significant transcriptional changes, which may explain the age-related loss of ischaemic tolerance [Moslehi, DePinho & Sahin 2012]. These transcriptional changes may be in part due to changes in the regulatory miRNA expression. Indeed, miRNAs have been shown to contribute to adverse outcomes and pathological remodelling following stress such acute myocardial infarction, transverse aortic constriction and are differentially expressed with ageing (Boon et al. 2014; Zhang, Azhar & Wei 2012; Bernardo et al. 2012). Furthermore, cardioprotective strategies such as IPC and IPOST have been shown to differentially activate the expression of several 'protectomiRs', where ablation of a single miRNA is sufficient to abolish the protective signalling (Yin, Salloum & Kukreja 2009; Varga et al. 2014). Similarly, cytoprotective responses such as caloric restriction also suppress circulating miRNA dysfunction that would otherwise occur with ageing (Dhabi et al. 2013).

In this study we investigated the expression of several candidate miRNAs suggested by the literature to be differentially expressed in response to ischaemia-reperfusion and ageing/senescence (Table 6.1). For example, members of the *miR-34* family have been implicated in myocardial infarction and transverse aortic constriction by interacting with genes involved in cell survival, angiogenesis, immune and cardiac function (Bernardo et al. 2012). Furthermore, miRNAs represent a novel class of pharmacological targets in the heart for restoring cardioprotection as knockdown of *miR-34a* has been shown to enhance I-R tolerance in the senescent mouse heart (Boon et al. 2013). Other prominent ageing-related miRNAs investigated in this study are shown in table 6.2. Besides investigating senescence and I-R related miRNAs, bioinformatics analysis was used to screen for candidate miRNAs predicted to target the caveolae-related transcripts *Cav3* and *Cavin1* which were previously shown to be down-regulated in the normoxic and post-ischaemic ageing hearts (Chapter 3).

Cardiac ageing is associated with a series of events including left ventricular hypertrophy, diastolic dysfunction, increased risk of atrial fibrillation, valvular degeneration and fibrosis which render the heart susceptible to stress such as ischaemia (Zhuo et al. 2014). AMI/I-R in both humans and animal models has been shown to lead to differential expression of miRNAs and is associated with adverse outcomes in patients (Bostjancic, Zidar & Glavac 2009; Gidlöf et al. 2008). More specifically, miRNA expression from cardiomyocytes, fibroblasts and endothelial cells contribute to pathogenesis of I-R injury by regulating genes involved in inflammation, fibrosis, apoptosis and angiogenesis (Sala et al. 2014). Although the expression of signature miRNAs involved in ischaemia-reperfusion are relatively well characterised in young hearts, expression of I-R related miRNAs have not been characterised in ageing models of post-ischaemic hearts with most age-related studies opting to characterise senescence-related miRNAs in aged and senescent hearts. Notable miRNAs reported to be affected by I-R and used in this study were *miR-1, miR-15, miR-20a, miR-21* and *miR-92a* (Boštjančic & Glavač 2014). As the expression of these prominent miRNAs remained uncharacterised in the ageing myocardium, we sought out to characterise several miRNA candidates involved in I-R injury by a variety of mechanisms (I.e. apoptosis/remodelling) which may be contribute to the decline of ischaemic tolerance in the ageing heart.

6.2 METHODS

6.2.1 Tissue samples and miRNA isolation.

Normoxic and post-ischaemic ageing male hearts (8, 16, 32 and 48 weeks old) were selected from the tissue biobank as described in Section 2.2.1. Hearts were removed from RNAlater preservative and dissected and homogenised as previously detailed in section 2.2. Total RNA containing miRNA fraction was extracted using miRNeasy columns (QIAxcel, Maryland) according to manufacturer's protocol. To determine yield and purity of RNA containing miRNA, NanoDrop ND-1000 (Thermo Fisher Scientific, MA, USA) was used.

6.2.2 cDNA synthesis

Following miRNA extraction, the NCode miRNA First-Strand cDNA Synthesis Kit (Life Technologies) was used to synthesise cDNA from 1.0 µg of cardiac total RNA containing the miRNA. Due to their small size (22nt) the mature miRNA first requires polyadenylation, secondly an oligo-dT adapter primer is used to prime the cDNA synthesis reaction. This adapter primer contains a unique proprietary sequence at its 5' end, which allows for amplification of cDNA using a complimentary universal primer paired with a primer specific to the miRNA sequence of interest (Figure 6.1).

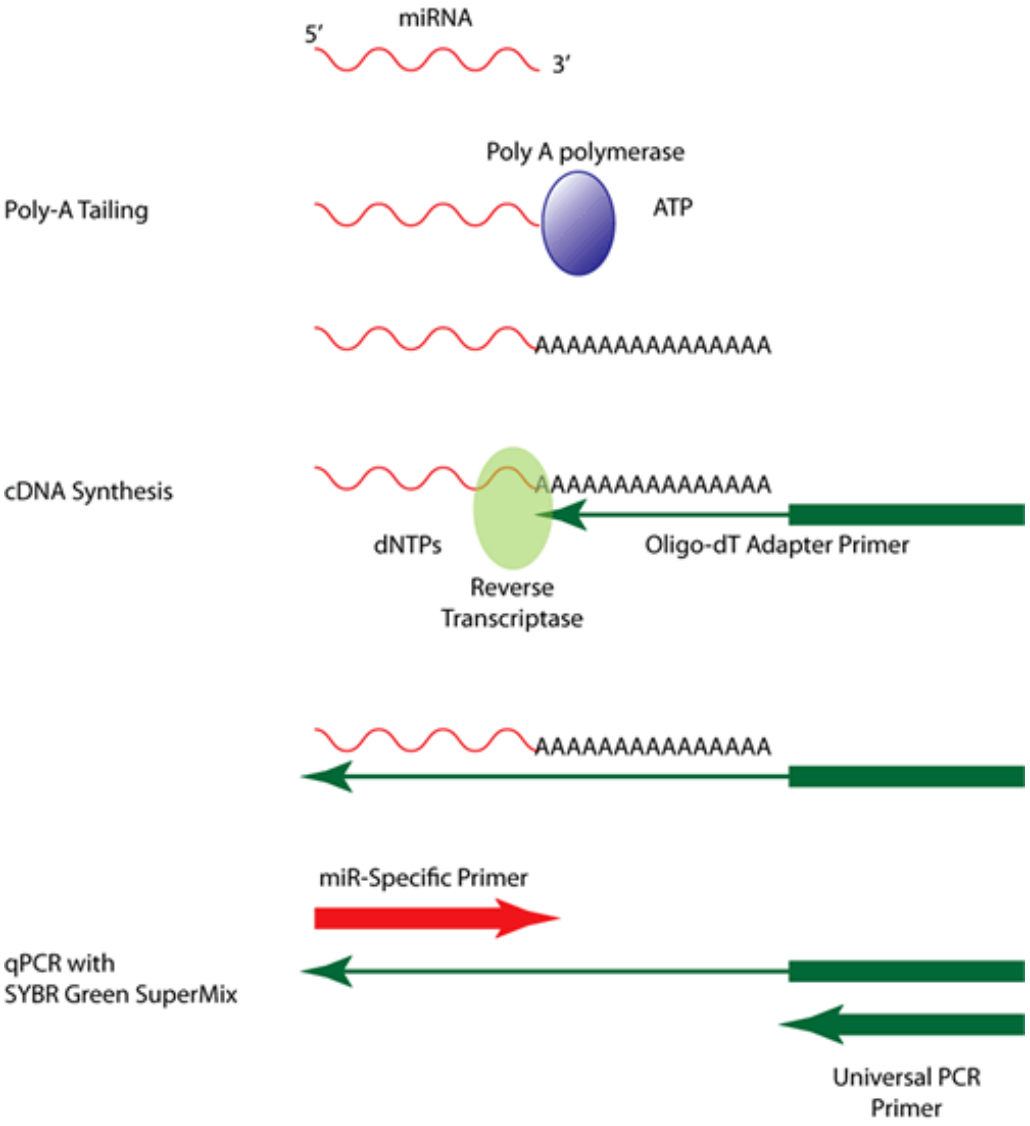


Figure 6.1 Overview of the cDNA synthesis procedure from miRNA (Image from: http://www.quantabio.com/product_mirna.php?base_id=95107)

6.2.3 RT-qPCR

Mature miRNA sequences were obtained from the miRBase database and used as the template to design the miRNA specific primer (Table 6.1). OligoAnalyzer (IDT) was used to design the miRNA primers at T_m of 58*C with BLAST used to ensure primer specificity to the miRNA target. Optimal qPCR cycling conditions consisted of an initial denaturation at 95°C for 3 minutes followed by 40 cycles of 95°C for 15 seconds and 58°C for 30 seconds. After the final PCR cycle, reactions underwent melt curve analysis to detect non-specific amplicons. We noted, unlike mRNA amplicons, miRNA amplicons displayed broad peaks, which were attributed to variable poly-A tailing of templates which may generate templates of few nucleotide differences in length. Reference gene selection for gene expression normalisation was performed using CFX-Manager (Bio-Rad) GeNORM analysis. The reference gene panel was composed of *miR-24*, *miR-26*, *miR-103*, *miR-191*, *RNU1A* and *RNU6,* with *miR-191* shown to be most stable in expression across both the normoxic and the post-ischaemic hearts (Figure 6.3A and 6.3B, respectively).

6.2.4 Bioinformatics analysis

For miRNA that showed significant changes, miRWalk 1.0 (Dweep *et al*. 2011) was used generate a list of validated mRNA targets. The validated mRNA targets were uploaded into Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/). The most relevant Gene Ontology (GO) terms generated by DAVID were represented with their gene clusters and number of genes. These genes were further used to generate a list of cardinally ordered top 5 cellular compartments by gene ontology (http://geneontology.org/).

Table 6.1 miRNA RT-qPCR primer sequences

Gene name	Gene symbol	NCBI GeneID	miRNA specific primer (5'-3')
Reference Genes			
microRNA 26a-1	*Mir26a-1*	387218	CAAGTAATCCAGGATAGGCTAA
microRNA 103-1	*Mir103-1*	723824	GCATTGTACAGGGCTATGA
microRNA 191	*Mir191*	387186	GAATCCCAAAAGCAGCTGA
U1a1 small nuclear RNA	*Rnu1a1*	19842	CGACTGCATAATTTGTGGTAG
U6 small nuclear RNA	*Rnu6*	19862	CCGGCAGCACATATACTAAAA
Ischaemia-Reperfusion			
microRNA 1a-1	*Mir1a-1*	387136	GTGGAATGTAAAGAAGTATGTATA
microRNA 15b	*Mir15*	387175	CAGCACATCATGGTTTACAA
microRNA 20a	*Mir20a*	387139	ACTAAAGTGCTTATAGTGCAG
microRNA 21a	*Mir21a*	387140	GCTTATCAGACTGATGTTGA
microRNA 24-1	*Mir24-1*	387142	CTCAGTTCAGCAGGAACAGA
microRNA 92-1	*Mir92-1*	751549	TATTGCACTTGTCCCGG
Senescence and Aging			
microRNA 22	*Mir22*	387141	AAGCTGCCAGTTGAAGA
microRNA 34a	*Mir34a*	723848	CAGTGTCTTAGCTGGTTGT
microRNA 34c	*Mir34c*	723932	CAGTGTAGTTAGCTGATTGC
microRNA 146	*Mir146*	387164	GAACTGAATTCCATGGGTT
microRNA 146b	*Mir146b*	751550	AGAACTGAATTCCATAGGCT
microRNA 378a	*Mir378*	723889	GGACTTGGAGTCAGAAGG
Caveolae-related			
microRNA 22	*Mir22*	387141	AAGCTGCCAGTTGAAGA
microRNA 92-1	*Mir92-1*	751549	TATTGCACTTGTCCCGG
microRNA 101b	*Mir101b*	724062	CAGTACTGTGATAGCTGAA
microRNA 485	*Mir485*	723875	CTGGCCGTGATGAATTC

6.3 RESULTS AND DISCUSSION

Normalisation of miRNA expression to unstable reference genes has been shown to drastically affect expression of miRNAs following I-R (Brattelid et al. 2011). The use of small nuclear RNAs including *RNU6* and *RNU1A* as reference genes still remains popular even without experimental assessment of stability. Furthermore, *RNU6* and *RNU1A* are small nuclear RNAs and thus do not accurately represent the miRNA population. Although the commonly used *RNU6* was stable in expression in the normoxic ageing hearts, post-ischaemic heart showed *RNU6* was unstable further highlighting the necessity of experimental validation between experimental conditions. In addition, other studies have shown *RNU6* to be up-regulated in aged hearts (Zhang et al. 2012). As shown in Figure 6.2, all miRNAs candidates assessed for stability showed stable expression in the ageing normoxic and post-ischaemic hearts, whereas *RNU6* and *RNU1A* were less suitable (> 1.0 M-value), specifically in the post-ischaemic heart (Figure 6.2B). Indeed, noticeable differences in *RNU6* expression can be seen in infarcted zones when compared to sham and border zones (Hullinger et al. 2012). GeNORM analysis identified *miR-191* as the most stably expressed miRNA in both the normoxic and post-ischaemic heart. Therefore, *miR-191* was chosen as the reference miRNA for normalisation in all subsequent RT-qPCR miRNA assays.

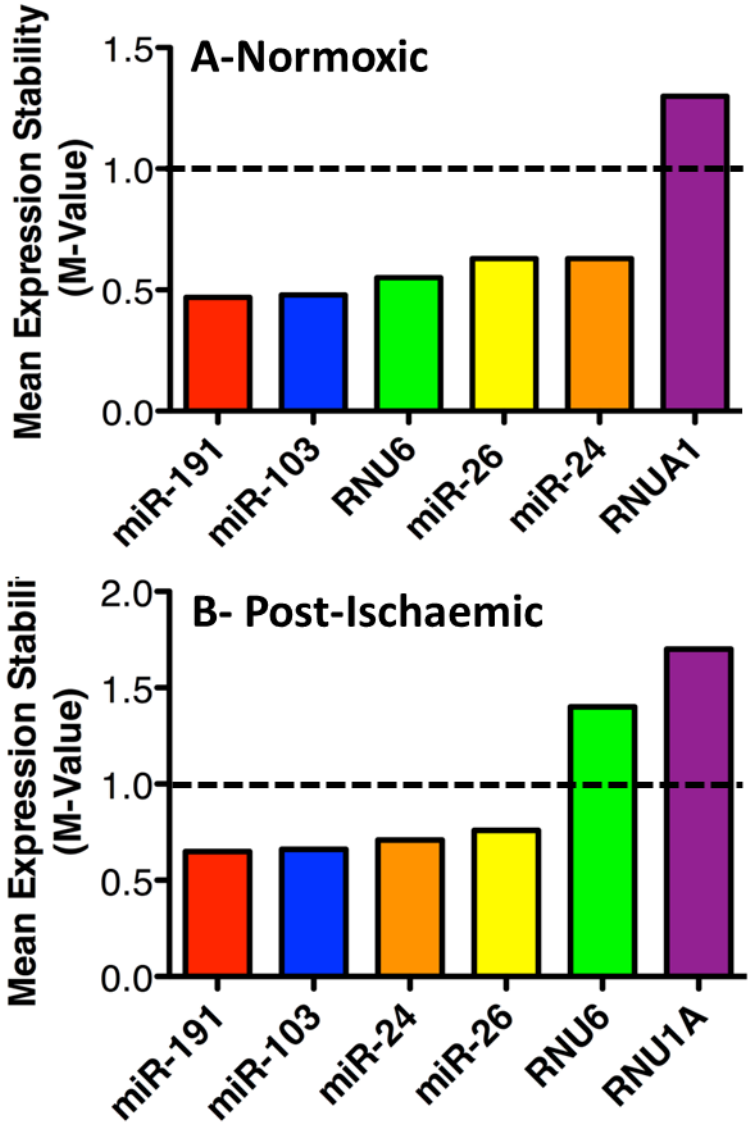


Figure 6.2 Bar graphs of expression stability of miRNA and small nuclear miRNAs normalisers in the normoxic and post-ischaemic ageing male hearts. Reference gene analysis in ageing **A)** normoxic and **B)** post-ischaemic mouse left ventricular tissue using miRNAs (*miR-24, miR-26, miR-103* and *miR-191*) and small nuclear RNA (*RNU6* and *RNU1A*) as candidates. The dotted line indicates the recommended threshold value of <1.0 for heterogeneous samples.

6.3.1 *Reference miRNA Stability Across Treatment Groups*

Similar to Chapters 3 and 4, based on the expression of *miR-191* reference gene in the normoxic and post-ischaemic hearts, a normalisation factor was applied to post-ischaemic hearts to allow for more uniform cross comparisons between these two groups. As shown in Figure 6.3, there were minimal differences in the baseline expression (p=0.92) of *miR-191* reference gene in the 8-week old hearts (1.1-fold) in both the normoxic and post-ischaemic groups.

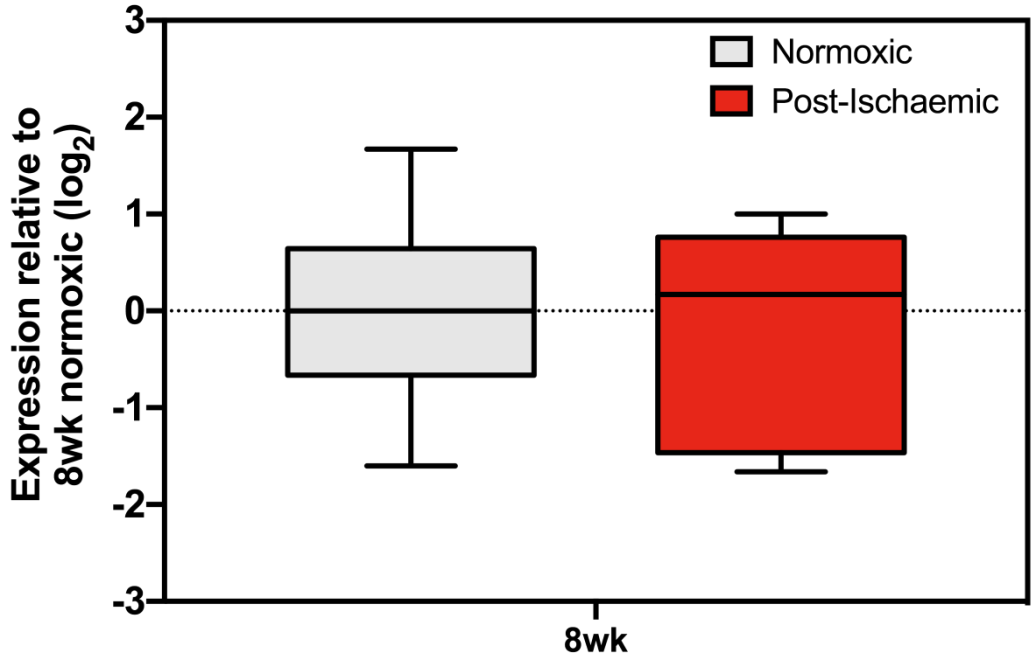


Figure 6.3 Assessment of *miR-191* stability relative to post-ischaemia in the normoxic murine heart. Shown are boxplots detailing gene expression changes determined by RT-qPCR for the *miR-191* reference gene in the male 8- and 48-week old heart following normoxic or post-ischaemic stress. Boxes indicate interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values. (n=6/group)

6.3.2 Ischaemia-Reperfusion Related miRNAs in the Normoxic & Post-Ischaemic Ageing Male Hearts

Of the six I-R related miRNAs assessed in this study, only *miR-15* and *miR-92a* showed significant age-related changes in expression. In the normoxic heart, *miR-15* and *miR-92a* showed significant 2.2-fold downregulation in the 8-week old hearts (Figure 6.3A and 6.3F, respectively). Surprisingly, the expression of these miRNA did not show changes in the post-ischaemic ageing hearts. Analysis by two-way ANOVA indicated significant interaction of age and ischaemia (Table 6.2) which can be seen in the 2.2-fold overexpression of *miR-15* and *miR-92a* in the 48-week old post-ischaemic hearts when compared to normoxic age-matched counterparts (Figure 6.4A and 6.4F, respectively). Whilst for *miR-24* this was borderline non-significant (P= 0.06) (Figure 6.4E) in the 48-week old post-ischaemic hearts by post-hoc analysis.

Table 6.2 Two-way analysis of variance of age and post-ischaemic effects on I-R-related transcript expression

Gene	Factor	Mean Square	F (DFn, DFd)	% of Total Variation	*P* value
miR-1	Age	0.057	0.47 (3,40)	3.0%	0.7030
	Ischaemia	0.311	2.54 (1.40)	5.3%	0.1190
	Age*Ischaemia	0.149	1.22 (3,40)	7.7%	0.3161
miR-15b	Age	0.319	3.73 (3,36)	11.3%	**0.0196**
	Ischaemia	2.046	24.42 (1,36)	24.7%	**< 0.0001**
	Age*Ischaemia	0.829	9.89 (3,36)	30.0%	**< 0.0001**
miR-20a	Age	3.400	6.86 (3,32)	25.6%	**0.0011**
	Ischaemia	8.902	17.96 (1,32)	22.3%	**0.0002**
	Age*Ischaemia	2.605	5.26 (3,32)	19.6%	**0.0046**
miR-21	Age	1.882	3.90 (3,32)	22.9%	**0.0175**
	Ischaemia	0.000	4.45e-004 (1,32)	0.1%	0.9833
	Age*Ischaemia	1.779	3.69 (3,32)	21.7%	**0.0218**
miR-24	Age	0.071	0.24 (3,40)	1.4%	0.8674
	Ischaemia	0.000	0.02 (1,40)	0.1%	0.8827
	Age*Ischaemia	1.264	4.26 (3,40)	23.9%	**0.0105**
miR-92a	Age	0.172	1.03 (3,39)	4.4%	0.3885
	Ischaemia	2.013	12.10 (3,39)	17.3%	**0.0013**
	Age*Ischaemia	0.988	5.94 (3,39)	25.5%	**0.0019**

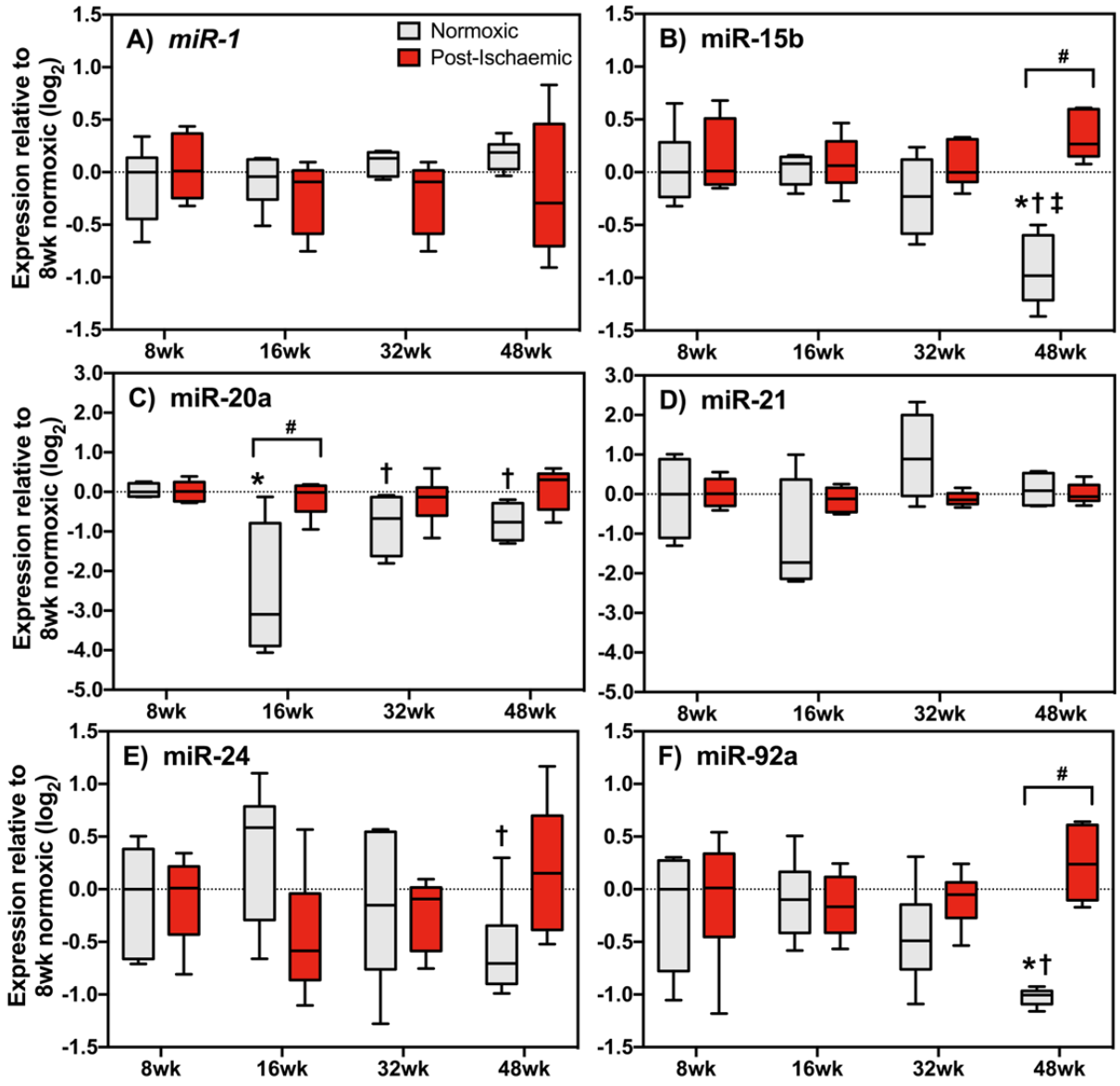


Figure 6.4 Age-dependent expression of I-R related miRNAs in normoxic male and post-ischaemic hearts. Shown are boxplots detailing gene expression changes determined by RT-qPCR for **A)** *miR-1*, **B)** *miR-15*, **C)** *miR-20a*, **D)** *miR-21*, **E)** *miR-24* and **F)** *miR-92a* expression in the ageing normoxic left ventricle. Boxes indicate the interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values. * $P < 0.05$ *vs.* 8-week old normoxic; † $P < 0.05$ *vs.* 16-week old normoxic; ‡ $P < 0.05$ *vs.* 32-week old normoxic; # P < 0.05 vs age-matched post-ischaemic group (indicated by brackets). (n=6/group).

The most characterised cardiac miRNA is the muscle-specific *miR-1*, which accounts for 40% of the total miRNA levels in the heart (Rao et al. 2009). Conflicting reports exist regarding the differential expression of *miR-1*, as it has been observed to be up- and down-regulated of *miR-1* as a result of I-R/AMI (Zhu & Fan 2012). We observed a minor trend in up-regulation of *miR-1* with ageing in the normoxic hearts, although not statistically significant. This observation is in agreement with human atrial biopsies of *miR-1* (Boon et al. 2013). It is questionable whether this trend of up-regulation would continue significantly in the senescent normoxic heart (72-weeks old) as others have shown that no changes in *miR-1* expression at this time point (Zhang, Azhar & Wei 2012). It is noteworthy that the samples used in this study were ventricular which have been shown to have unique transcriptional profile (Ng, Wong & Tsang 2010). IPOST (vs. sham) has been shown to significantly induce expression of several miRNAs including *miR-1, miR-15b, miR-21, miR-24* and *miR-26* (Tu et al. 2013). These IPOST-related miRNAs showed no changes in expression in the post-ischaemic ageing hearts used in this study. Although *miR-15* and *miR-92a* were decreased in expression in the 48-week old normoxic heart (Figure 6.4B and 6.4F), there was no differential expression observed in their post-ischaemic counterparts (Figure 6.4B and 6.4F). However, the up-regulated expression of *miR-15b* found in this study (Figure 6.4B) is not consistent with the induced expression of *miR-15b* in IPOST and its role as an antiangiogenic miRNA.

Bioinformatic functional clustering of *miR-15b* validated targets are mostly involved in transcription and cell proliferation processes (Table 6.4). As previously discussed, although *miR-15b* and *miR-92a* showed no aged-related changes in expression in post-ischaemic hearts, it is possible that they may be induced in expression relative to their normoxic counterparts. Indeed, ischaemia results in elevated levels of *miR-15b* and *miR-92a* (Hullinger et al. 2012; Frank et al. 2012). To further support the notion that *miR-15b* and *miR-92a* may be induced in the 48-week old post-ischaemic hearts relative to normoxic counterparts, inhibition of these miRNAs following I-R has been shown to be cardioprotective (Bonauer et al. 2009; Hinkel et al. 2013). Consistent with these reports, we observed up-regulation of *miR-15b* and *miR-92a* in the 48-week old post-ischaemic hearts when compared to their normoxic age-matched counterparts (Figure 6.4). *MiRNA-92a* belongs to the miR 17-92 cluster which is antiangiogenic, whilst *miR-15b* has also been to be antiangiogenic (Liu et al. 2012). Consistent with this, mRNA targets for both *miR-15b* and *miR-92a* are enriched in

transcriptional and cell proliferation-related clustering as shown in Tables 6.3 and 6.4, respectively. More specifically, *miR-15b* is a positive regulator of many transcriptional activities as well as being implicated in cell differentiation. For example, *miR-15* can halt cardiomyocyte division by repressing cell cycle genes such as checkpoint kinase 1 (Porello et al. 2011). Induction of this miRNA is consistent with the compromised division of cardiomyocytes in the aged hearts which display adverse remodelling following I-R (as previously discussed in Section 1.6). Similarly, *miR-92a* is implicated to be involved in nucleic acid-related processes as well as cell proliferation. In terms of cell proliferation, *miR-92a* has been shown to be involved in controlling angiogenesis by targeting proangiogenic proteins, such as the integrin subunit alpha5 (Bonauer et al. 2009). Similarly, functional annotation implicates *miR-92a* involvement in the regulation of cellular response to stress and blood vessel morphogenesis (Table 6.4).

We also observed a 1.8-fold induction of *miR-24* in the 48-week old post-ischaemic hearts relative to their age-matched counterparts, although this was borderline non-significant (P= 0.06). *MiR-24* appears to be a pro-apoptotic miRNA and similar to *miR-92a* in its role as a critical regulator of endothelial cell apoptosis and angiogenesis. In the heart, *miR-24* overexpression has been shown to trigger endothelial apoptosis and impairs angiogenesis whilst *miR-24* inhibition shown to increase vascularisation and improve cardiac function after MI (Meloni et al. 2013; Fiedler et al. 2011). The induction of this miRNA in the 48-week old post-ischaemic hearts is coincident with the loss of cardioprotection in these hearts and its role as a pro-apoptotic miRNA. Taken together, induction of both these I-R responsive miRNAs (*miR-15b*, *miR-24* and *miR-92a*) may contribute to loss of ischaemic tolerance and adverse remodelling following I-R in the 48-week old hearts and their induction in the middle-hearts is also consistent with their role as pro-apoptotic miRNAs.

Table 6.3 Enrichment of *miR-15b* targets using DAVID functional annotation clustering showing regulatory networks implicated by the mRNA targets of this miRNA.

GO Domain	GO Term	Number of Genes	Gene Symbols	Top 5 Cellular Compartments
Cell Component	Transcription factor complex	8	*CLOCK, HIF1A, POU5F1, SMAD2, SMAD3, SMAD4, SMAD5, SMAD7*	CHOP-C/EBP complex SMAD protein complex RNA polymerase II transcription factor complex Nuclear transcription factor complex Transcription factor complex
Molecular Function	Sequence-specific DNA binding	11	*CEBPB, DDIT3, FOXA2, ISL1, NEUROD1, NKX2-2, NKX6-1, PDX1, POU5F1 RELA, ZEB2*	
Biological Process	Negative regulation of cell proliferation	10	*ADORA2A, BCL2, CDKN1A, FGF2, HMOX1, IFITM3, PDX1, SMAD4, TNF, TRP53*	

Table 6.4 Enrichment of *miR-92a* targets using DAVID functional annotation clustering showing regulatory networks implicated by the mRNA targets of this miRNA.

GO Domain	GO Term	Number of Genes	Gene Symbols	Top 5 Cellular Compartments
Cell Component	Transcription factor complex	16	*CREB1, CTNNB1, E2F1 E2F2, E2F6, GATA6, HOXA9, NKX2-1, NKX2-5, POU5F1, RBL2, SMAD4, SOX2, SPIB, YY1, ZEB1,*	Cell Cell part Intracellular part Membrane-bounded organelle Organelle
Molecular Function	Cyclin-dependent protein kinase inhibitor activity	5	*CASP3, CDKN1C, CDKN1A, CDKN2A, CDKN1B*	
	Negative regulation of molecular function	11	*ADORA1, CASP3, CDKN1A, CDKN1B, CDKN2A, DHCR24, HIPK3, HMOX1, MYC, NR4A1, PDCD4*	
Biological Process	Regulation of cellular response to stress	3	*CDKN2A, HIPK3, PDCD4*	
	Blood vessel morphogenesis	16	*ACVR1, B4GALT1, BMP4, CTGF, CTNNB1, CXCR4, DICER1, FGF10, FGF2, FGFR1, HMOX1, MAPK14, NKX2-5, PTEN, TDGF1, TGFBR2*	

6.3.3 Ageing and Senescence-Related miRNAs

Of the six senescence and ageing associated miRNAs investigated only *miR-22* (Figure 6.5A) and *miR-378* (Figure 6.5F) showed significant differential expression. More specifically, *miR-22* was down-regulated 1.8-fold in the 48-week old normoxic heart relative to 8-week old controls. Two-way ANOVA indicated that significant interaction of ageing and I-R was present only for *miR-22* (Table 6.5) as shown by the 2.3-fold induction of *miRNA-22* (Figure 6.5A) in the 48-week old post-ischaemic hearts compared to age-matched normoxic hearts. Whilst an age-related decline in *miR-378* was observed, with both 16-week (1.5-fold) and 48-week old (1.7-fold) normoxic hearts demonstrating significant down-regulation relative to 8-week. Conversely, in the post-ischaemic ageing heart, only *miR-378* repression in expression that was borderline non-significant (P= 0.06) (Figure 6.5F). Specifically, there was a decrease in expression at both 32 and 48-week (1.2-fold) old hearts relative to 8-week old male hearts (Figure 6.5F).

Table 6.5 Two-way analysis of variance of age and post-ischaemic effects ageing and senescence-related transcript expression.

Gene	Factor	Mean Square	F (DFn, DFd)	% of Total Variation	*P* value
miR-22	Age	0.729	2.90 (3,39)	13.2%	**0.0460**
	Ischaemia	0.766	3.05 (1, 39)	4.6%	0.0885
	Age*Ischaemia	1.258	5.01 (3.39)	22.8%	**0.0049**
miR-34a	Age	0.104	0.42 (3,38)	2.9%	0.7388
	Ischaemia	0.538	2.35 (1.38)	5.4%	0.1332
	Age*Ischaemia	0.208	0.84 (3,38)	5.7%	0.4797
miR-34c	Age	0.863	2.57 (3,38)	16.2%	0.0687
	Ischaemia	0.365	1.09 (1,38)	2.3%	0.3039
	Age*Ischaemia	0.093	0.28 (3,38)	1.8%	0.8413
miR-146a	Age	0.228	1.31 (3,39)	8.4%	0.2835
	Ischaemia	0.197	1.14 (1,39)	2.4%	0.2931
	Age*Ischaemia	0.166	0.96 (3,39)	6.1%	0.4208
miR-146b	Age	0.694	2.58 (3.40)	12.7%	0.0667
	Ischaemia	2.244	8.35 (1,40)	13.7%	**0.0062**
	Age*Ischaemia	0.436	1.62 (3,40)	8.0%	0.1996
miR-378	Age	1.118	9.24 (3,38)	35.7%	**0.0001**
	Ischaemia	0.701	5.80 (1,38)	7.5%	**0.0210**
	Age*Ischaemia	0.195	1.61 (3,38)	6.2%	0.2033

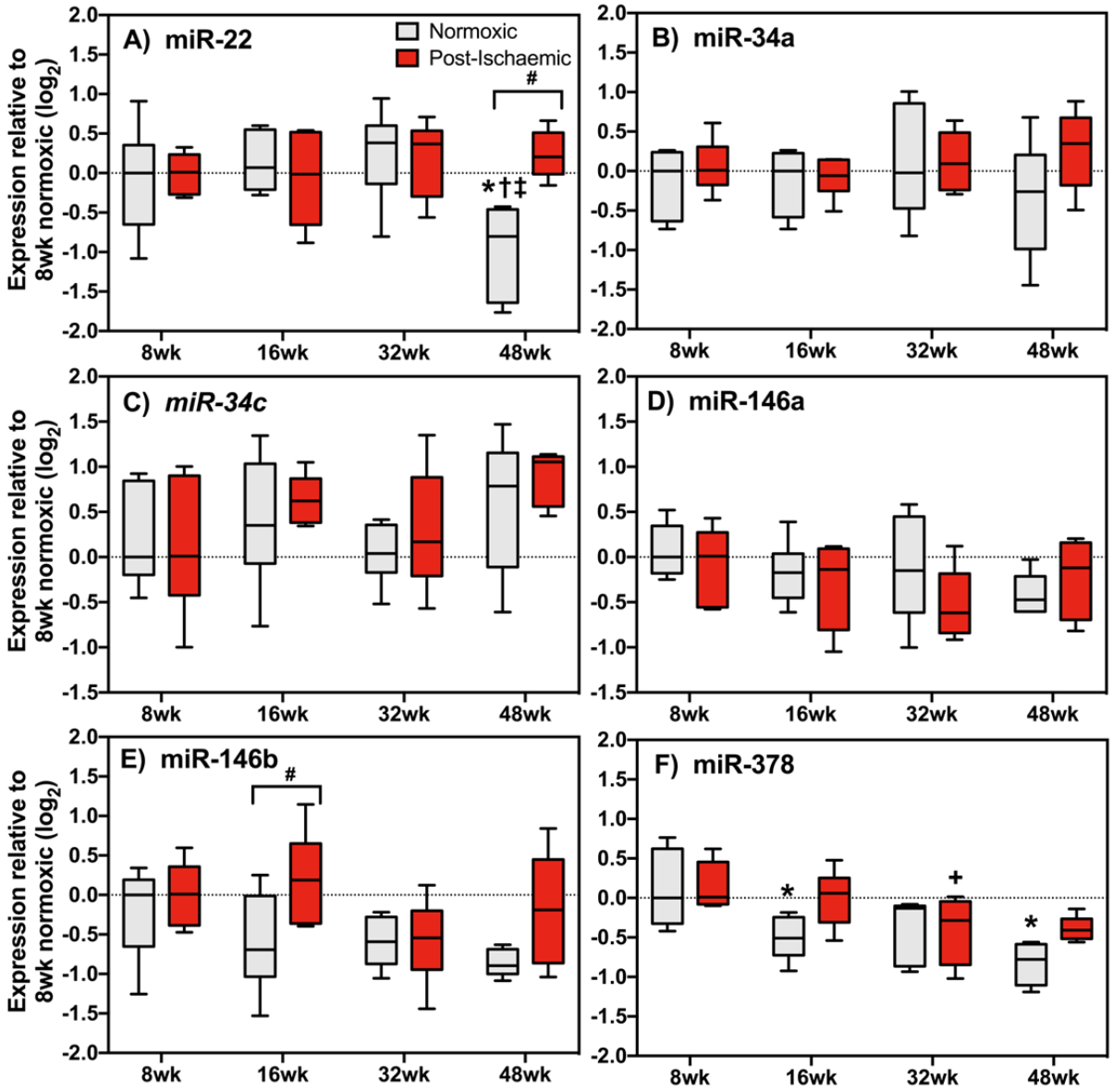


Figure 6.5 Age-dependent expression of ageing/senescence related miRNAs in normoxic and post-ischaemic male hearts. Shown are boxplots detailing gene expression changes determined by RT-qPCR for **A)** *miR-22,* **B)** *miR-34a,* **C)** *miR-34c,* **D)** *miR-146a,* **E)** *miR-146b* and **F)** *miR-378* in the normoxic (grey boxes) and post-ischaemic (red boxes) heart. Boxes indicate the interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values. *, $P < 0.05$ vs. 8-week old normoxic; †, $P < 0.05$ vs. 16-week old normoxic; ‡, $P < 0.05$ vs. 32-week old normoxic; +, $P<0.05$ vs. 8-week old post-ischaemic; # $P < 0.05$ vs age-matched post-ischaemic group (indicated by brackets). (n=6/group).

Ageing is associated with changes in mRNA expression, which has been shown to impact a variety of cellular pathways, including apoptosis, remodelling and angiogenesis in both normoxic and post-ischaemic hearts (Ashton et al. 2006). Similarly, differential expression of miRNAs is observed with cellular senescence, which supresses variety of cellular pathways such as cell proliferation, differentiation and metabolism (Dhabhi et al. 2011). Senescence is characterised by complex miRNA-related processes such as reduced telemetric length, cell division, mitochondrial metabolism and increased fibrosis in the heart (Kovacic et al. 2011; Jazbutyte et al. 2013; Boon et al. 2013). Deep sequencing of aged hearts (>74 weeks old) has been shown to identify differential expression of 65 miRNAs, of which 55% were up-regulated and 45% down-regulated compared to young counterparts (Zhang, Azhar & Wei 2012). Whilst several deep sequencing datasets are available for the aged heart, detailed functional clustering/annotation has yet to be performed to identify affected mRNA networks. This is perhaps owing to the lack of validated mRNA targets of the differentially expressed miRNAs (Zhang, Azhar & Wei 2012; Zhang et al. 2014; Boon et al. 2013). Based on literature analysis, several miRNA candidates (*miR-34a, miR-34c, miR-22, miR-15, miR-146a, miR-146b* and *miR-378*) related to ageing/senescence were investigated in the normoxic and post-ischaemic aging heart. Members of the miR-34 family have become promising strategies as the inhibition of miR-34 family has been shown to ablate age-related loss of cardioprotection as well as resistance to moderate transverse aortic constriction (Boon et al. 2013; Bernardo et al. 2014).

Of the miRNAs assessed, only *miR-378* showed significant down-regulation in both groups (Figure 6.5F) although the down-regulation of *miR-378* was borderline non-significant in the post-ischaemic hearts. However, other studies have reported an up-regulation of *miR-378* in 43-week old normoxic hearts when compared to 4-week old counterparts (Knezevic et al. 2012). Similarly, we observed a decline in *miR-22* expression at 48-weeks old (Figure 6.5A). Whilst others have observed an age-related increase in *miR-22* in the aged and senescent heart (Jazbutyte et al. 2013). The reason for this discrepancy is unknown, however noticeable differences between the studies are differences in species (C57/Bl6 mouse compared to Sprague-Dawley rat), tissue analysed (left ventricular vs whole heart), treatment (heart explant vs Langendorff) and reference gene used for normalisation (*RNU6*). Targets of *miR-378* have been suggested to include *Igf1r* and *Grb2*. Activation of Igf1r by Igf1 has been shown

to be cardioprotective (Buerke et al. 1995). Grb2 has complex interactions with tyrosine kinases such as Egfr and Fgfr2 by regulating their internalisation and phosphorylation status (Ahmed et al. 2013; Yamazaki et al. 2002). In the heart, $Grb2^{-/+}$ mice show reduced phospho-p38 and phospho-JNK when challenged with transverse aortic constriction (Zhang et al. 2003). As discussed in Section 1.6.5, the aged heart shows reduced expression of phospho-p38 MAPK. Bioinformatic analysis also implicates *miR-378* involvement in telomeres, telomerase, cellular ageing, and immortality (Table 6.7), with telomeres reduced and telomerase activity decreased in the aged heart as previously detailed in Section 1.6.1. However, *miR-378* has been shown to be down-regulated with hypoxia and its overexpression has been shown to result in reduced cell death following hypoxia (Fang et al. 2012). These observations are consistent with the ischaemic-intolerant phenotype observed in the ageing hearts used in this study which displayed significant repression of *miR-378* in the 32 and 48-week old (borderline non-significant) post-ischaemic hearts.

Although *miR-22* was down-regulated in the 48-week old post-ischaemic heart, similar to *miR-15b* and *miR-92a*, *miR-22* was induced in expression relative to its age-matched normoxic counterpart in the 48-week old hearts (Figure 6.5A). Overexpression of *miR-22* has been shown to contribute to cellular senescence and migration of cardiac fibroblasts (Jazbutyte et al. 2013). Consistent with this, bioinformatics analysis of *miR-22* suggested that it is mostly involved in transcription related process (Table 6.6). *Bona fide* target of *miR-22* has been shown to be *mimecan* although the effects of increased *mimecan* expression on human ventricular remodelling are currently controversial (Jazbutyte et al. 2013; Motiwala et al. 2013).

Expression of members within the *miR-34* family has been shown to be dysregulated with ageing in the heart and the brain (Boon et al. 2013; Li et al. 2011a). In the heart, *miR-34* expression is dysregulated with cardiac stress such as myocardial infarction and transverse aortic constriction (Bernardo et al. 2012). In aged hearts (74 weeks old), *miR-34a* expression shows 2.0-fold induction (vs 8 week old) and is inversely correlated with its targets *Pten* and *Pnuts* expression (Boon et al. 2013). Inhibition of *miRNA-34a* in the aged hearts has been shown to enhance ischaemic tolerance and recovery when compared to age matched wild-types (Boon et al. 2013). Although we did not find changes in *miR-34a* expression in the hearts used in this study, other groups have shown that 32-week old normoxic hearts show

significant up-regulation of *miR-34a* expression which correlated inversely with significantly reduced *Pnuts* expression in these hearts (Ito, Yagi & Yamakuchi 2010). Future studies should investigate the expression of *Pnuts* and whether its expression is affected with ageing. Several of the miRNAs candidates related to ageing and senescence used in this study only *miR-378* showed significant down-regulation in the 48-week old post-ischaemic hearts. However, when compared to 48-week old normoxic hearts, there was significant up-regulation of *miR-22* in the 48-week old post-ischaemic hearts. It has been suggested that miRNA dysregulation may start at mid-life and does not become apparent until the aged phenotype (Li et al. 2011). To further support this notion, time-course analysis of *miR-34a* expression does not show noticeable changes in 18-week old mouse brain with *miR-34a* expression changes becoming apparent in 52-78 week old hearts (Li et al. 2011). Similarly, in the mouse brain, aged-related reduction in mitochondria-related transcripts does not become apparent until 72-96 weeks of age (Manczak et al. 2005). In *C. elegans*, miRNA dysregulation occurs at the post-reproductive phase past mid-life (Ibáñez-Ventoso et al. 2006).

Table 6.6 Enrichment of *miR-22* targets using DAVID functional annotation clustering showing regulatory networks implicated by the mRNA targets of this miRNA.

GO Domain	GO Term	Number of Genes	Gene Symbols	Top 5 Cellular Compartments
Cell Component	Chromosome	6	*DNMT3B, H1FO, H2AFX, H2AFZ, SYCP3, TRP53,*	Intracellular membrane-bounded organelle Intracellular organelle Membrane-bounded organelle Organelle Nucleus
Molecular Function	Transcription factor activity	11	*ESR1, ETS1, KLF6, MYC, NFAT5, POU5F1, PPARA, RERE, SOX11, SP1, TRP53*	
Biological Process	Transcription	15	*ANG, ESR1, ETS1, IGHMBP2, KLF6, MAX, MCM7, MYC, NFAT5, POU5F1, PPARA, RERE, SOX11, SP1, TRP53*	

Table 6.7 Enrichment of *miR-378* targets using DAVID functional annotation clustering showing regulatory networks implicated by the mRNA targets of this miRNA.

GO Domain	GO Term	Number of Genes	Gene Symbols	Top 5 Cellular Compartments
Cell Component	Chromosomal part	6	*DNMT3B, H1FOO, H2AFX, H2AFZ, SYCP3, TRP53*	Nucleus Organelle lumen Intracellular organelle lumen Nuclear part Nuclear lumen

6.3.4 Bioinformatic prediction of caveolae-related miRNA

Bioinformatic prediction using miRWalk was used to select the top miRNAs targeting *Cav3* and *Cavin1*. Several public databases are available, which predict miRNA binding sites to target genes by the use of varying algorithms dependent on the site (Dweep, Sticht, & Norbert 2013). Although the use of such databases has widely helped study miRNA-mRNA interactions, they are also believed to generate false positive rates of >0.3 or specificity that is often less than 70% (Zheng et al. 2013). In this study, we used the miRWalk database as it incorporates other popular miRNA prediction databases (i.e. TargetScan and miRanda) which have been suggested to minimise the possibility of introducing false positives and/or negatives. Following database analysis, *miR-101b, miR-22* and *miR-92a* were top predictors for *Cav3* (Table 6.9), while for *Cavin1* the top predictors were *miR-485* and *miR-22*.

Following RT-qPCR, *miR-101b*, *miR-22* and *miR-92a* showed decreased transcript expression in the 48-week old normoxic heart (Figure 6.6). Expression of these miRNAs did not correlate inversely with the reduced *Cav3* expression previously shown in the 16-, 32-, and 48-week old hearts (Figure 3.3C). Similarly, no corresponding miRNA expression was found in the post-ischaemic hearts for the miRNAs targeting *Cav3* (Figure 3.4C) with these miRNAs also not showing any changes in expression (Figure 6.6). More specifically, as *Cav3* in the normoxic heart is down-regulated as early as 16-weeks, we did not observe any miRNAs that fit this profile. For the miRNAs targeting *Cavin1*, *miR-22* and *miR-481* also did not show inverse patterns of expression in the normoxic heart and post-ischaemic hearts (Figure 6.7A and Figure 6.7B, respectively).

Previous studies have shown that caveolin expression can be controlled by miRNA processes as *Cav1* has been shown to be a validated target of *miR-802* (Lin et al. 2011). However, the top miRNA predicators for *Cav3* and *Cavin1* did not show inverse correlation to their respective mRNAs with only *miR-92a* and *miR-22* showing significant change in the normoxic heart although this also did not correlate inversely with *Cav3* expression. As shown in table 6.9, note the varying consensus of miRNA databases on *Cav3* target prediction, i.e. *miR-92a* is not predicted to target *Cav3* according to miRDB or RNA22 whilst the other three databases suggest *miR-92a* interacts with *Cav3*. Besides miRNA-mediated mechanisms, other possible mechanisms that may influence gene expression include transcriptional factors, which

influence gene expression at the transcriptional level. In the case of *Cav3*, myogenin has been shown to interact with the *Cav3* promoter while Id2 has been shown to inhibit promoter activation by myogenin (Biederer et al. 2000; Martínez-Moreno et al. 2007). Other mechanisms that have been shown to modulate gene expression include complex interaction of epigenetic changes such as DNA methylation and histone acetylation/deacetylation (Gonzalo 2010).

Table 6.8 Bioinformatic prediction of miRNAs targeting *Cav3* using miRWalk, which uses several other popular databases to predict miRNA targets of a given gene. Green highlight; Predicted target from database, red highlight; not predicted target from database; yellow highlight; sum of all database predictions.

Gene Name	MicroRNA	miRanda	miRDB	miRWalk	RNA22	Targetscan	SUM
Cav3	*Mmu-miR-101b*	1	1	1	0	1	4
Cav3	*Mmu-miR-22*	1	1	1	0	1	4
Cav3	*Mmu-miR-92a-2*	1	0	1	0	1	3
Cav3	*Mmu-miR-412*	1	0	1	0	1	3
Cav3	*Mmu-miR-464*	1	0	1	0	1	3

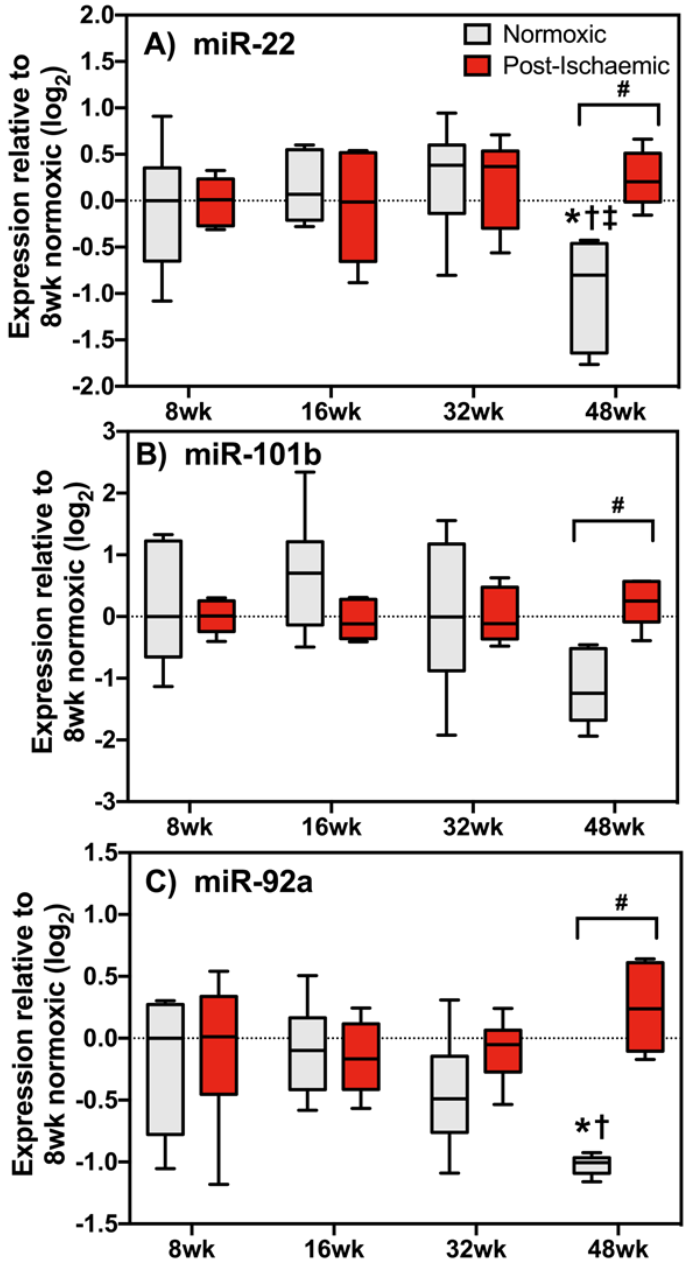


Figure 6.6 Age-dependent expression of *Cav3*-related miRNA transcripts in normoxic and post-ischaemic male hearts. Shown are boxplots detailing gene expression changes determined by RT-qPCR for **A)** *miR-22,* **B)** *miR-101b* and **C)** *miR-92a* targeting *Cav3* in the normoxic (grey boxes) and post-ischaemic (red boxes) heart. Boxes indicate the interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values. *, $P < 0.05$ vs. 8-week old normoxic; †, $P < 0.05$ vs. 16-week old normoxic; ‡, $P < 0.05$ vs. 32-week old normoxic; #, $P < 0.05$ vs age-matched post-ischaemic group (indicated by brackets). (n=6/group).

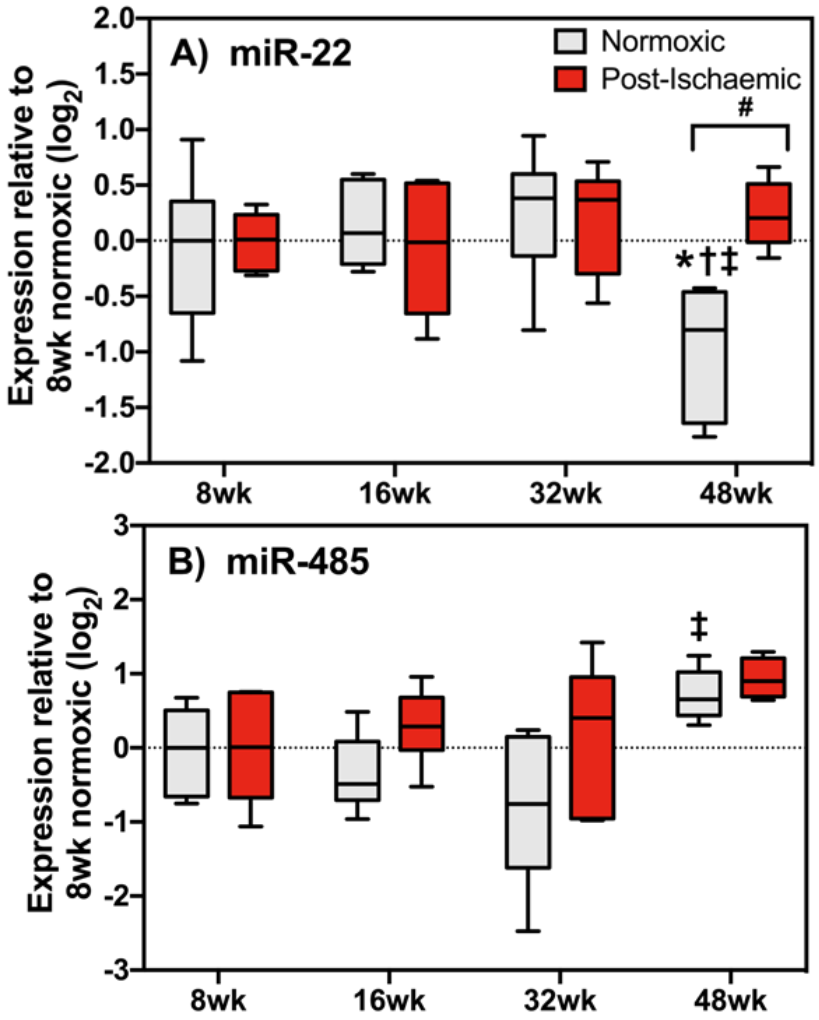


Figure 6.7 Age-dependent expression of *Cavin1*-related miRNA transcripts in normoxic and post-ischaemic male hearts. Shown are boxplots detailing gene expression changes determined by RT-qPCR for **A)** *miR-22* and **B)** *miR-485* targeting *Cavin1* in the normoxic (grey boxes) and post-ischaemic (red boxes) heart. Boxes indicate the interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values. *, $P < 0.05$ vs. 8-week old normoxic; †, $P < 0.05$ vs. 16-week old normoxic; ‡, $P < 0.05$ vs. 32-week old normoxic; #, $P < 0.05$ vs age-matched post-ischaemic group (indicated by brackets). (n=6/group).

6.4 CONCLUSION

In this study, we investigated the expression of several miRNAs genes in the ageing normoxic and post-ischaemic mouse heart. The miRNAs chosen were based on previous studies, which reported their association with ischaemia and ageing/senescence. Additionally, bioinformatics analysis was used to predict miRNA candidates that may regulate *Cav3* and *Cavin1* transcripts, as these were previously shown to be altered in the ageing heart (Chapter 3). Although most miRNA investigations use high-throughput methods such as next-generation sequencing or microarrays, we selected a candidate gene approach employing RT-qPCR exclusively. This method has been shown to be reliable platform for detecting miRNA expression (Mestdagh et al. 2014; Wang 2009). Here, we also show the feasibility of using SYBR Green-based method using online tools and universal priming which can quantitate miRNAs that are relatively low in expression in the heart (e.g. *miR-34c*), despite the universal RT priming method used in this experiment criticised for low specificity which we did not observe in this study (via capillary electrophoresis of amplicons) (Chugh & Dittmer 2012).

Transcriptional regulation is mechanistically complex and multifaceted, involving the use of mRNA transcriptional factors, which can induce miRNA and mRNA expression and conversely use of miRNAs to suppress mRNA expression. Furthermore, the recent discovery of "sponge miRNA" which bind to multiple miRNAs adds to the stunning complexity of gene regulatory network within the cell (Matkovich, Hu, & Dorn 2013; Hansen et al. 2013). The aims of this study were to investigate the expression of several miRNAs candidates based on ageing/senescence, ischaemia-reperfusion and miRNAs targeting caveolae transcripts (Table 6.2). Of the miRNAs assessed related to ageing/senescence, only *miR-378* showed consistent down-regulation in both the normoxic and post-ischaemic heart ageing hearts. The reduced *miR-378* in the 32- and 48-week old hearts has mixed implications, as Grb2 reduction would be consistent with the loss of phospho-p38 MAPK signalling in the aged heart. Of the miRNAs associated with I-R, only *miR-15* and *miR-92a* showed significant down-regulation in the normoxic hearts but not in the post-ischaemic hearts ageing. However, in the 48-week old post-ischaemic hearts *miR-15* and *miR-92a* was elevated vs. age-matched normoxic counterparts. The induction of *miR-15* and *miR-92a* was consistent with the stress-intolerant 48-week old hearts. In addition, several of the miRNAs were investigated were implicated to be 'protectomiRs' (*miR-1, miR-15, miR-21, miR-24* and *miR-26*), shown to be up-regulated in

IPOST methods. There were no protectomiRs changes in the ageing post-ischaemic hearts. However, when compared to their 48-week old normoxic counterparts, we observed up-regulation of *miR-15* and *miR-24* which was not consistent with the suggested role of these miRNAS as "protectomiRs". Lastly, bioinformatic prediction of miRNAs targeting caveolae transcripts *Cav3* and *Cavin1* was employed to see whether the reduction in these transcripts was miRNA-related process in the ageing hearts. There was no inverse expression correlation observed with miRNAs that were predicted to target *Cav3* and *Cavin1* in the ageing hearts (Figure 6.5 and Figure 6.6), although it remains a possibility other miRNAs and/or transcriptional factors may be involved in the down-regulation of *Cav3* and *Cavin1*.

It must be noted that the middle-aged phenotype time points used in this study is typically different to the 'aged' phenotype (>70 weeks old) is typically used in ageing studies (Li et al. 2011). As discussed in Section 6.3.2, age-related perturbations in miRNA expression appear during the aged time point (>70 weeks old), which has also been the predominant time point used in majority of ageing studies (Boon et al. 2013; Jazbutye et al. 2013). In addition, I-R protocols (i.e. Langendorff vs left anterior descending artery ligation) also vary between studies, with assessment of cardiac function following myocardial infarction being up to 8-weeks post treatment in some cases as demonstrated by Bernardo and colleges (2012). Therefore, one of the limitations of this study is due to the shorter reperfusion protocol (60 minutes). Due to this limitation, cardiac remodelling and other processes may require longer reperfusion times to become apparent (as seen in Bernardo et al. 2012), which may not have been detected with the Langendorff model used in this study. Another limitation of this study is the possibility of confounding gene expression due to use of left ventricular tissue, which contains considerable amounts of fibroblasts and endothelial cells (Banerjee et al. 2007). For example, *miR-34a* has been shown to be present in both cardiomyocytes and endothelial cells (Boon et al. 2013).

7. Chapter Seven

Plasma membrane repair-related transcript expression in the normoxic and post-ischaemic ageing heart

ADDENDUM

Contributions to Chapter 7:

Can Kiessling:

- Experimental design
- All gene expression & protein expression analysis
- Data analysis
- Author of the chapter

Kevin Ashton:

- Assistance with experimental design and interpretation of results
- Editing of chapter

Melissa Reichelt:

- Heart perfusions

7.1 INTRODUCTION

As previously detailed in Section 1.7, the plasma membrane plays an integral part in cardioprotective signalling (Boengler, Schulz & Heusch 2009). By compartmentalising many cardioprotective receptors such as GPCRs the A_1AR, δ-opioid receptor, and RTKs such as EGFR and their subunits, activation of cardioprotective receptors in the plasma membrane has been shown to enhance survival signalling leading to enhanced ischaemic tolerance and recovery (Lasley 2012; Patel et al. 2006). Ischaemic stress has been shown to results in multiple tiny breaks in the plasma membrane that, without immediate repair results in irreversible cell injury leading to necrosis (Jennings et al. 1983). Indeed, commonly used indicators of cell death detect changes in membrane permeability, such as LDH release or changes in membrane folding, including annexins. Injury to the plasma membrane frequently occurs in cells subjected to high levels of mechanical activity including skeletal and cardiac muscle cells. These cells experience constant mechanical stress, where up to 25% of cell wounding has been observed as a result (McNeil & Steinhardt 2003; Clarke et al. 1995). Cells which undergo a second disruption of the plasma membrane have been shown to repair the damage more rapidly than the initial wound, thus suggesting the presence of active processes (Togo et al. 1999). Alterations in plasma membrane repair mechanisms are also associated with variety of diseases including muscular dystrophy, cancer invasion and in type 1 and 2 diabetes (He et al. 2012; Jaiswal et al. 2014; Howard et al. 2011).

Most plasma membrane repair mechanisms are Ca^{2+}-dependant and require the concerted effort of several cytoskeletal and plasma membrane proteins (Andrews, Almeida & Corette 2014). Although the plasma membrane can reseal naturally *via* thermodynamic processes, this recovery is limited (McNeil & Kirchhausen 2005). Thus, the cell possesses a 'toolkit' of repair proteins, including ferlins, annexins, calpains, acid-spingomyolinases and the tripartite motif family of proteins, with representative members are shown in Table 7.1. Depending on the size of the wound, repair to the damage sites has been shown to involve complex events including: 1) remodelling of the cortical skeleton around the wound site, 2) changes in endocytic/exocytic events around the wound, 3) patch formation and 4) closure of the wound (Corrotte et al. 2013). To orchestrate this task, membrane repair involves the use of several different classes of proteins with differing roles such as proteases (calpains), calcium

sensing/binding proteins (annexins), E3 ubiquitin protein ligases (TRIMs), and spingomyolinases (SMPDs).

Membrane-repair mechanisms have been shown to be reliant on caveolae and their coat proteins for normal trafficking and targeting to sites of injury (see Table 7.1 and Figure 7.1) (Cai et al. 2009; Zhu et al. 2011). Preservation of sarcolemmal integrity has been shown to be a noticeable feature of Cav3-overexpression and IPC, which may enhance these membrane-repair mechanisms in the heart (Tsutsumi et al. 2008; Murry et al. 1990). Indeed, membrane-repair mechanisms are crucial mediators of cardioprotective mechanisms such as IPC and IPOST as ablation of plasma membrane repair gene *Trim72* results in loss of cardioprotection in murine hearts (Zhang et al. 2011; Cao et al. 2010). Furthermore, membrane-repair-related machinery are also implicated in the repair of the sarcomeric contractile apparatus which may be relevant to cardioprotection in terms of recovery following I-R (Cooper & Head 2014; Inserte et al. 2009). In this study, we investigated the expression of membrane repair-related transcripts, which are involved in several different stages of the membrane and sarcomeric repair which are typically associated with caveolae and remain largely uncharacterised in the ageing heart.

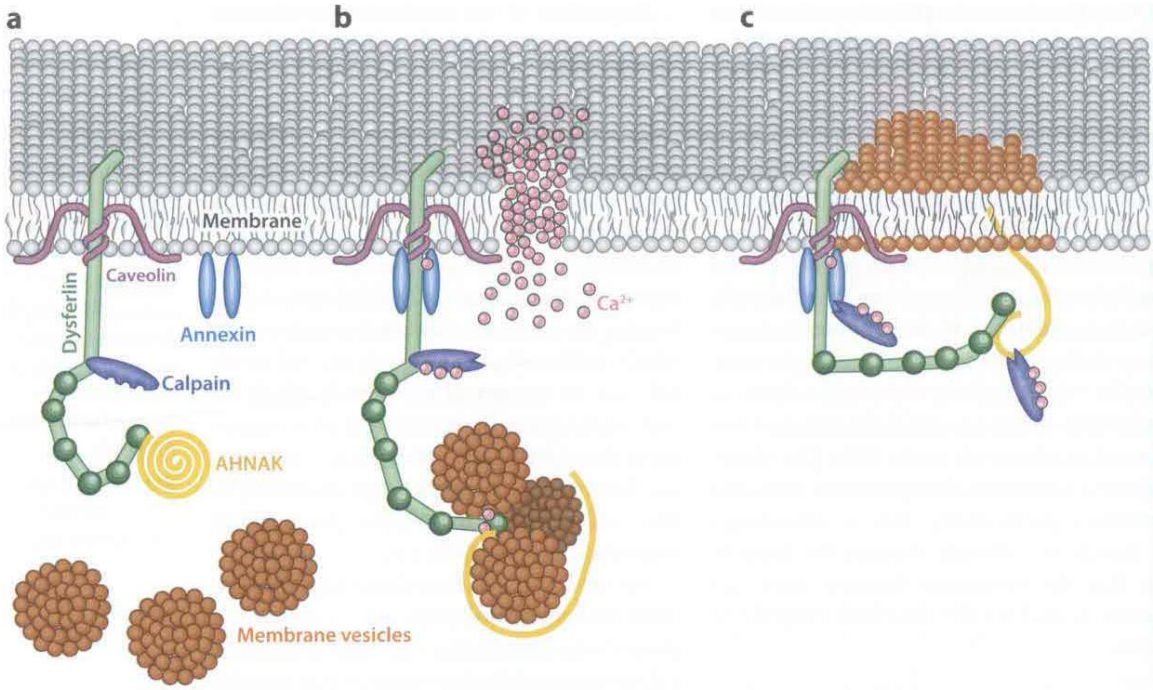


Figure 7.1 Current plasma membrane repair signalling paradigm A) Membrane repair requires the concerted effort of several classes of proteins, which interact with caveolins and caveolae. **B)** Damage to the membrane causes influx of calcium ions, which causes activation of calpains, annexins and other calcium sensitive proteins which help incorporate vesicles fusion to 'patch' the membrane. **C)** Within seconds the calcium concentration returns to normal levels when membrane integrity is restored, leading to inactivation of the repair complex. (Taken from Wallace & McNally 2009).

Table 7.1 Representative members used in this study of membrane-related repair family proteins consisting of Ferlins, Annexins, Calpains, Spingomyolinases and Tripartite motif. It must be noted that this is not a comprehensive list, with other notable families such as ESCRT, Synaptotagmins and other genes also participate in the membrane repair processes (Jimenez et al. 2014).

Family (Symbol)	**Members**	**Localises/Interacts with Caveolins/Cavins**	**Sarcomeric Repair**
Ferlin	Dysferlin, Myoferlin	+ -	+ -
Annexin (Anxa)	Annexin-1 Annexin-5 Annexin-6	- - +	+ - -
Calpain (Capn)	Calpain-1 Calpain-2 Calpain-3	- + -	+ - +
Acid-Spingomyolinases (Smpd)	Spingomyolinase-1 Spingomyolinase-2	? -	- -
Tripartite motif (Trim)	Trim-54 Trim-72	- +	+ -

7.2 METHODS

Langendorff perfusions were carried out on hearts (n=8-10/per group) from 8, 16, 32 and 48 week old female mice as previously described in Section 2.1.1. Briefly, 20 minutes of global normothermic ischaemia was followed by 60 minutes of reperfusion whilst normoxic hearts were reperfused for 80 minutes.

RNA isolation and cDNA synthesis from normoxic and post-ischaemic left ventricular tissue was carried out as described Section 2.2.1.

RT-qPCR was used to analyse expression of *Trim*, *Ferlin*, *Anxa*, *Capn* and *Smpd* transcripts (Table 7.2) in ageing hearts as detailed in Section 2.3.1. Selection of a stable reference gene was carried out according to Section 2.3.2. Normalisation of post-ischaemic gene expression to normoxia was carried out according to Section 2.4.

Western immunoblotting was carried to confirm validate the relationship between transcript and protein expression changes for Trim72 according to Section 2.5.

Statistical analysis was conducted on data as per Section 2.6 with statistical significance accepted for $P<0.05$.

Table 7.2 RT-qPCR primer sequences for plasma membrane repair transcripts.

Gene name	Gene symbol	NCBI GeneID	Forward primer (5'-3')	Reverse primer (5'-3')
Tripartite motif-containing				
Trim54	*Trim54*	58522	CAGCCGCAGACAGAAGCAAC	CTTGCGCTCCTCCAAAACCG
Trim72	*Trim72*	434246	GCCTCAAGACACAGCTTCC	GCACCGCTACAGTCTTCTC
Annexins				
Annexin1	*Anxa1*	16952	CCTACCCTTCCTTCAATGTATCC	TGCTTCATCCACACCTTTAACC
Annexin5	*Anxa5*	11747	GACAAGTACATGACCATATCAGGA	GAATAGACTTCACGACAGCCA
Annexin6	*Anxa6*	11749	CGCATACAAAGACGCCTATGAG	AAATCCTCGCTCACAACATCG
Ferlins				
Dysferlin	*Dysf*	26903	CTGCGATGTTTCCCTCTATGTG	CGTCCACTTCTATTTCTCCTCC
Myoferlin	*Myof*	226101	CTGAACGAAGCCTCCTGAC	GTGATGCCGCCTTATCTCC
Calpains				
Calpain1	*Capn1*	12333	AATCTCCCAAACTCCCCTTCCC	CGCTTCTTCTGTACTTGGGCTG
Calpain2	*Capn2*	12334	AGAGTTCTAGCCAAACGCC	CTCCCATCTTCATCCAGCA
Calpain3	*Capn3*	12335	TCCATTGACGATGGCACGAA	TCCGTGCAATCAACTCCCCC
Sphingomyelinases				
sphingomyelin phosphodiesterase 1, acid lysosomal	*Smpd1*	20597	CCTTAACCCTGGCTACCGA	CAAGATGTAGGTCTCGTGGT
sphingomyelin phosphodiesterase 2, neutral	*Smpd2*	20598	GCTCTGATGATGGCTGTACC	AGAGACTGCCTTGTAAAGCAC

7.3 RESULTS & DISCUSSION

7.3.1.1 Transcriptional analysis of trim expression

RT-qPCR was used to assess the expression of several families of proteins involved in plasma membrane repair and sarcomere maintenance as listed in Table 7.1.

Trim74 expression was stable with ageing in both normoxic and post-ischaemic hearts (Figure 7.2A). In the normoxic hearts, *Trim54* showed 2-fold overexpression with ageing in the 16, 32 and 48-week old hearts when compared to 8-week old (Figure 7.2B). This trend was also present within the post-ischaemic hearts which showed ~1.3-fold induction of *Trim54* in the 32 and 48-week old hearts vs 8-week old post-ischaemic hearts (Figure 7.2B). These findings are further supported by two-way ANOVA analysis which identified significant effect of age and ischaemia whilst the significant interaction of age and ischaemia was borderline non-significant (Table 7.3). When compared to age-matched normoxic counterparts, there was reduction (~1.4-fold) of *Trim54* transcript in the ageing post-ischaemic hearts which was significant in the 16 and 48-week old post-ischaemic hearts (Figure 7.2B)

Table 7.3 Two-way analysis of variance of age and post-ischaemic effects on Trim transcript expression

Gene	Factor	Mean Square	F (DFn, DFd)	% of Total Variation	*P* value
Trim72	Age	0.3373	3.02 (3,39)	17.8%	**0.0413**
	Ischaemia	3.285e-005	2.94e-004 (1,39)	0.1%	0.9864
	Age*Ischaemia	0.096	0.86 (3,39)	5.0%	0.4716
Trim54	Age	1.384	33.60 (3,36)	55.4%	**< 0.0001**
	Ischaemia	1.302	31.63 (1,36)	17.4%	**< 0.0001**
	Age*Ischaemia	0.108	2.62 (3,36)	4.3%	0.0654

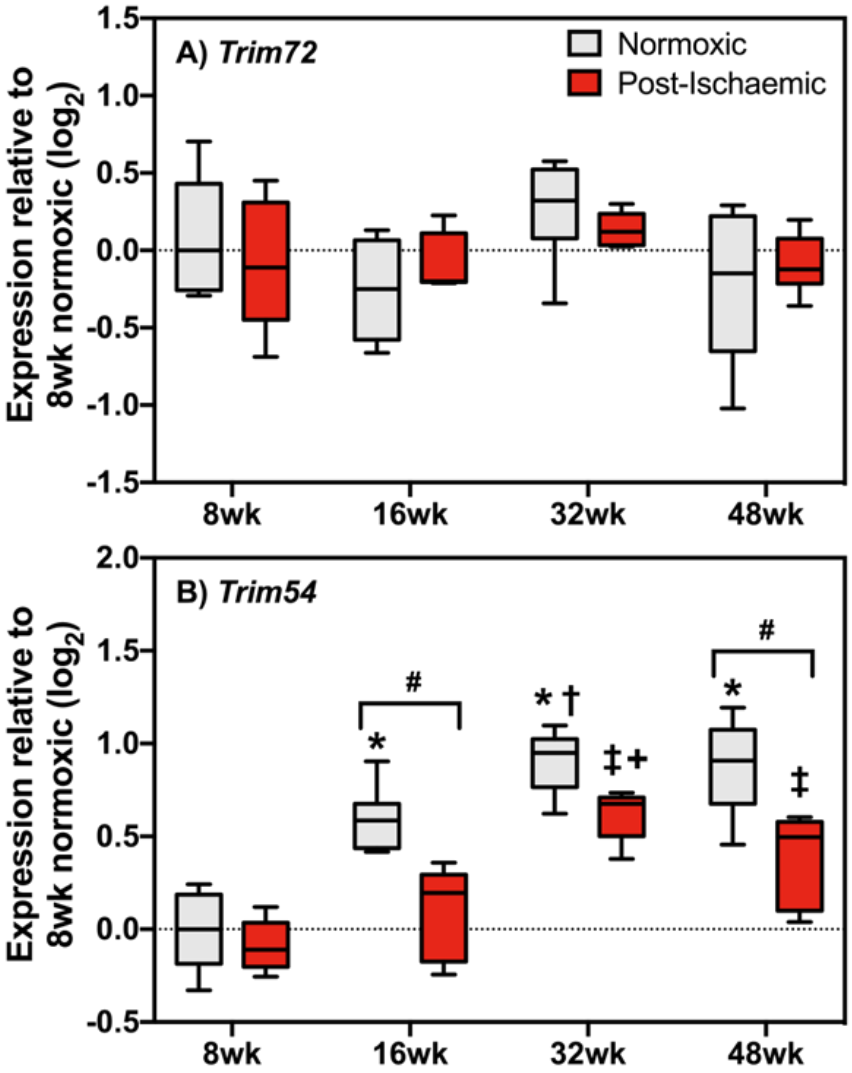


Figure 7.2 Age-dependent expression of Trim transcripts in normoxic and post-ischaemic male hearts. Shown are boxplots detailing expression changes determined by RT-qPCR for **A)** *Trim72* and **B)** *Trim54* in the normoxic (grey boxes) and post-ischaemic (red boxes) heart. Boxes indicate interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values. * $P < 0.05$ vs 8-week old normoxic; † vs 16-week old normoxic; ‡ $P < 0.05$ vs 8-week old post-ischaemic; + $P < 0.05$ vs 16-week old post-ischaemic; # $P < 0.05$ vs age-matched post-ischaemic group (indicated by brackets). (n=6/group).

7.3.1.2 Transcriptional analysis of trim expression

As Trim72 is known to interact with Cav3 and showed a possible down-regulation in the normoxic 48-week old hearts, immunoblotting was used to verify the *Trim72* transcript and protein expression. As shown in Figure 7.3A, normoxic 48-week old hearts showed a 2-fold down-regulation of Trim72 that was borderline non-significant (P= 0.07) whilst post-ischaemic 48-week old hearts did not show this age-related decrease (Figure 7.3B).

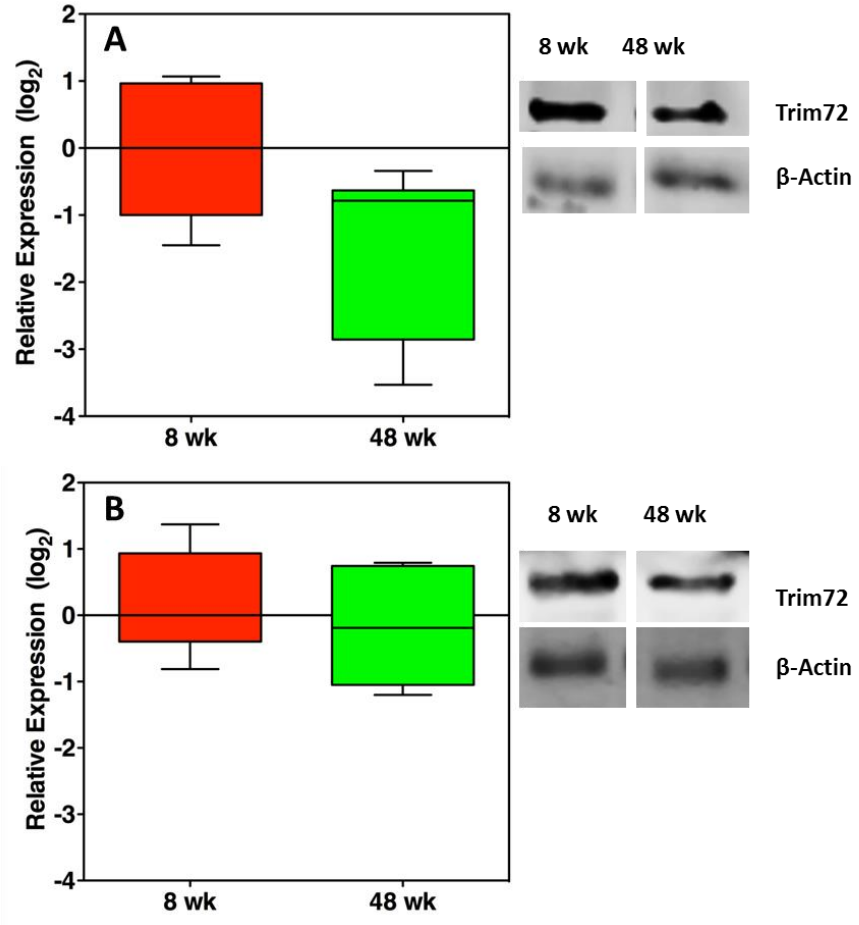


Figure 7.3 Age-dependent expression of Trim72 protein in normoxic and post-ischaemic male hearts. Validation of transcript and protein expression changes for left ventricular Trim72 in **A)** normoxic and **B)** post-ischaemic ageing hearts. Boxes indicate the interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values. To the right of the graphs representative immunoblot images are shown for Trim72 and the β-Actin loading control. (n=6/group)

The TRIM family of proteins consists of numerous members, which have been shown to regulate a variety of cellular functions including immunity, cell cycle, autophagy and membrane repair (Alloush & Weisleder 2013; Micale et al. 2012; Mandell et al. 2014). Targeting of Trim72 to injury sites requires Cav3 and Cavin1 as knockdown of either *Cav3* or *Cavin1* results defective trafficking of Trim72 to membrane injury sites (Cao et al. 2010; Zhu et al. 2011). The role of Trim72 in membrane repair is thought to be in recruitment of repair components, including dysferlin and annexin5. Trim72 is also though to serve as a scaffold to facilitate membrane repair (Cao et al. 2010). It has been proposed that the oligomerisation of Trim72 at the injury site provides anchorage for fusion of intracellular vesicles to the damaged sarcolemma. In support of this is the observation that Trim72-deficient mice lack intracellular vesicle accumulation following membrane injury when compared to wild-types (Cai et al. 2009). Besides its role in membrane repair mechanisms, Trim72 may also be involved in activation of survival kinases, as Trim72-deficient hearts demonstrate reduced phospho-Akt and Erk1/2 activation following IPC (Cao et al. 2010). Conversely, Trim72-overexpression results in significant activation of phospho-Akt and Erk1/2 (Cao et al. 2010). The enhanced survival kinase expression in these hearts may be due to Trim72's role as an inhibitor of IRS1, an upstream activator of PI3k. Indeed, Trim72 and IRS1 are associated together as shown by co-immunoprecipitation (Lee et al. 2010). Owing to its success in animal models, recombinant Trim72 has been suggested to be a therapeutic for IHD although its efficacy in the aged heart is unknown and may be affected due to reduction of Cav3, Cavin1 and caveolae in the aged post-ischaemic hearts (Chapter 3) (Liu et al. 2015; Peart et al. 2014).

Trim54 (aka Murf3) is an E3 ubiquitin protein ligase that plays a role in degradation of sarcomeric proteins, stabilization of microtubules and myogenesis (Olivé et al. 2015). In the heart, Trim54 together with Murf1 and Calpain1, help maintain contractility by marking damaged and aggregated proteins such as FHL2 and γ-filamin for degradation (Fielitz et al. 2007). To further highlight their importance, *Trim54*-deficiency results hypertrophic cardiomyopathy and decreased fractional shortening compared to wild-types (Fielitz et al. 2007). Following I-R *Trim54*-deficiency has been shown to results in cardiac rupture in 77% of *Trim54*-deficient hearts whilst this dramatic rupture was not observed in sham and wild-type mice (Fielitz et al. 2007). The molecular mechanisms leading to this cardiac rupture after MI remain unknown.

7.3.2.1 Transcriptional analysis of annexin expression

Transcriptional analysis of *Anxa1* and *Anxa6* did not show changes in expression in the normoxic and post-ischaemic ageing heart (Figure 7.4A-C, respectively). For *Anxa5*, highly significant effect of age was identified using two-way ANOVA analysis (Table 7.4). *Anxa5* showed a significant 2.6-fold down-regulation in the 16 week-old normoxic hearts with the 32 and 48-week old normoxic hearts showing 1.6-fold decrease (Figure 7.4C). Whilst this age-related reduction of *Anxa5* was also present in the post-ischaemic ageing hearts, it was only significant in the 16-week old hearts vs 8-week old hearts (Figure 7.4B).

Table 7.4 Two-way analysis of variance of age and post-ischaemic effects on annexin transcript expression

Gene	Factor	Mean Square	F (DFn, DFd)	% of Total Variation	*P* value
Anxa1	Age	0.325	1.77 (3,36)	10.3%	0.1696
	Ischaemia	0.854	4.66 (1,38)	9.0%	**0.0373**
	Age*Ischaemia	0.249	1.35 (3,38)	7.9%	0.2712
Anxa5	Age	1.146	10.25 (3,36)	42.5%	**< 0.0001**
	Ischaemia	0.289	2.58 (1.36)	3.6%	0.1167
	Age*Ischaemia	0.099	0.89 (3,36)	3.7%	0.4570
Anxa6	Age	0.014	0.39 (3,40)	2.7%	0.7591
	Ischaemia	0.002	0.08 (1,40)	0.2%	0.7891
	Age*Ischaemia	0.027	0.77 (3,40)	5.3%	0.5182

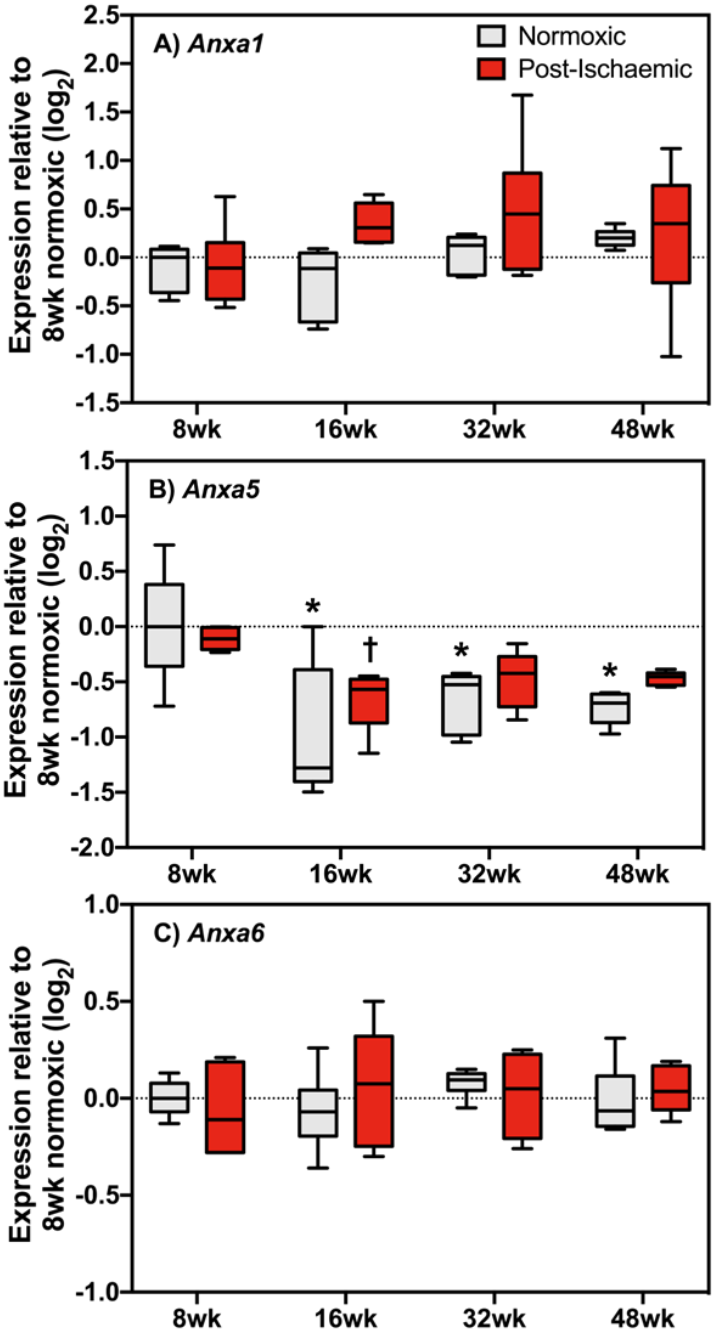


Figure 7.4 Age dependent expression of annexin transcripts in normoxic and post-ischaemic male hearts. Shown are boxplots detailing expression changes determined by RT-qPCR for **A)** *Anxa1,* **B)** *Anxa5* and **C)** *Anxa6* in the normoxic (grey boxes) and post-ischaemic (red boxes) heart. Boxes indicate the interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values. * $P < 0.05$ vs 8-week old; † vs 8-week old post-ischaemic. (n=6/group).

The annexin family consist of twelve ubiquitously expressed genes that are Ca^{2+} and phospholipid-binding proteins. Annexins participate in a variety of cellular processes, including the regulation of membrane dynamics, cell migration, calcium handling and apoptosis (Monastyrskaya 2009; Camors, Monceau & Charlemagne 2005). We investigated expression of *Anxa1*, *Anxa5* and *Anxa6* as they have been implicated in formation of repair patches following plasma membrane injury (McNeil et al. 2006; Bouter et al. 2011; Swaggart et al. 2014). Of the annexins transcripts investigated, only *Anxa5* showed a significant change in expression with ageing. *Anxa5* was down-regulated with ageing in the normoxic hearts (Figure 7.4B). Anxa5 has been shown to participate in membrane repair by the formation of two-dimensional ordered arrays of trimers on phosphatidylserine exposing membranes (Bouter et al. 2011). The authors proposed that the two-dimensional array formed strengthens the membrane and prevents the expansion of the tear (Bouter et al. 2011). Interestingly, *Anxa5*-deficient mice are fertile and show normal development with no changes in blood plasma biochemical parameters (Brachvogel et al. 2003). However, the authors did not investigate muscle histology or markers of oncosis/necrosis (e.g. CK, LDH or troponin), although it is possible that *Anxa5*-deficiency does not result in impairment plasma membrane integrity by itself (Brachvogel et al. 2003). It is unclear whether repair-patch formation in the aged hearts is significantly affected partly owing to lack of kinetic investigations. In end-stage human heart failure, expression of Anxa5 has been shown to be induced and cluster in the cytosol of cardiomyocytes with a lack of organisation (Song et al. 1998; Benevolensky et al. 2000; Matteo & Moravec 2000). It is interesting to speculate whether this lack of organisation could be from the reduction of Cav3/Cavin1-mediated targeting to sites of injury in the failing hearts (Ratajczak et al. 2003).

7.3.3.1 Transcriptional analysis of ferlin expression

Dysf did not show any changes in expression with ageing in the normoxic and post-ischaemic hearts (Figure 7.5A). In the normoxic hearts, *Myof* showed 1.4-fold up-regulation in the 48-week old hearts vs 16 and 32-week old hearts (Figure 7.5B). Post-ischaemic *Myof* expression was unaffected with ageing (Figure 7.5B).

Table 7.5 Two-way analysis of variance of age and post-ischaemic effects on ferlin transcript expression

Gene	Factor	Mean Square	F (DFn, DFd)	% of Total Variation	*P* value
Dysf	Age	0.307	1.94 (3,38)	11.2%	0.1401
	Ischaemia	0.278	1.75 (1,38)	3.4%	0.1938
	Age*Ischaemia	0.337	2.12 (3,38)	12.3%	0.1138
Myof	Age	0.692	3.93 (3,40)	17.7%	**0.0151**
	Ischaemia	0.188	1.07 (1,40)	1.6%	0.3077
	Age*Ischaemia	0.807	4.58 (3,40)	20.6%	**0.0075**

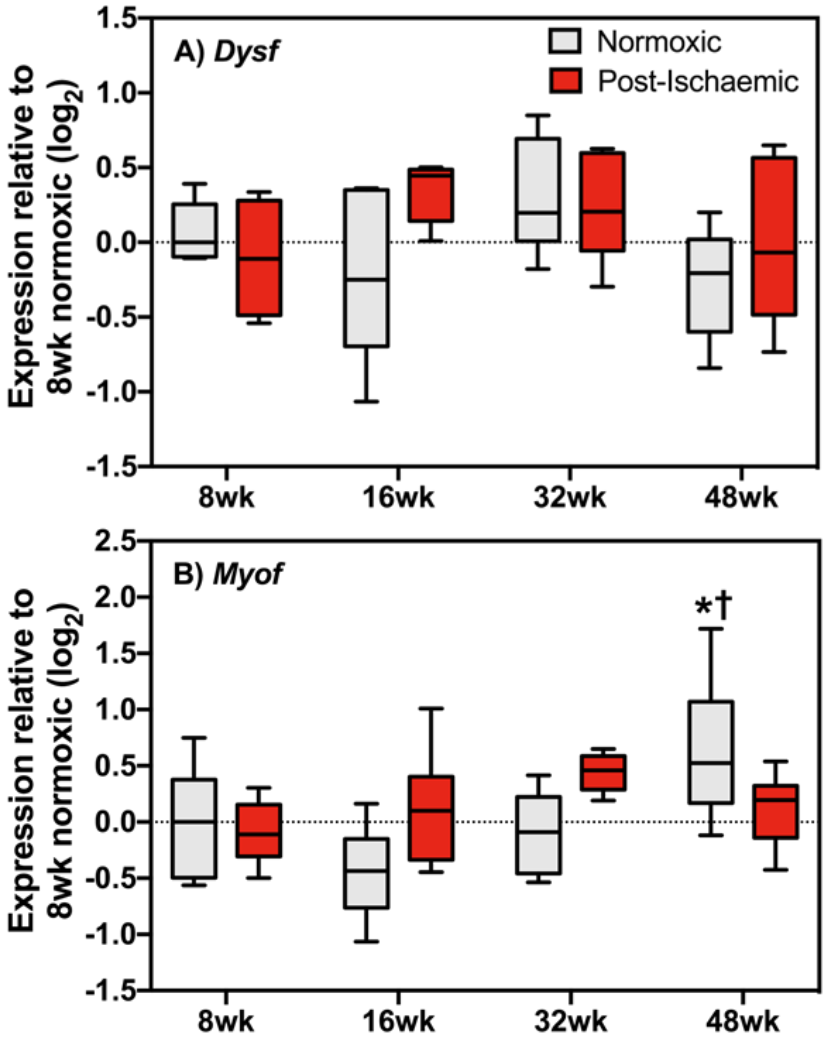


Figure 7.5 Age dependent expression of ferlin transcripts in normoxic and post-ischaemic male hearts. Shown are boxplots detailing expression changes determined by RT-qPCR for **A)** *Dysf* and **B)** *Myof* in the normoxic (grey boxes) and post-ischaemic (red boxes) heart. Boxes indicate the interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values. †, $P < 0.05$ vs. 16-week old normoxic; ‡, $P < 0.05$ vs 32-week old normoxic. (n=6/group).

The ferlin family of genes consists of the highly homologous dysferlin (Dysf), myoferlin (Myof), otoferlin (Otof) and fer-like family. The ferlin family is part of the dystrophin complex with disruption of this complex associated with cardiomyopathy and muscle diseases (i.e. Miyoshi myopathy) as a result of plasma membrane instability (Heydemann & McNally 2007). Dysferlin-deficient mice display defective plasma membrane sealing with progressive muscular dystrophy (Bansal et al. 2003). Dysf interacts with several membrane repair related proteins in the sarcolemma including Cav3, annexins and calpains (Kobayashi et al. 2012). Cav1 and Cav3-deficient cells show abnormal Dysf trafficking, similar to aberrant Dysf localisation in caveolinopathies suggests that caveolins may be important in targeting of Dysf to the membrane (Hernández-Deviez et al. 2008; Matsuda et al. 2001). More specifically, mutations in Cav3 result in retention of Dysf-Trim72 complexes in the Golgi apparatus suggesting caveolins help target plasma membrane machinery to sites of injury (Cai et al. 2009).

The role of Dysf seems to involve, in conjunction with acid-spingomyolinase targeting of vesicular and lysosome fusion to injury sites as Dysf-deficient mice show reduced tethering of lysosomal exocytosis (Defour et al. 2014). In addition, Dysf-deficient mice show Anxa1 and Anxa2 accumulation at the site of injury (Lennon et al. 2003). Upon injury, Calpain-mediated cleavage of Dysf into Mini-dysferlin$_{C72}$ patches appears to be the first-response accumulating at the exposed edge of the membrane (Lek et al. 2013). Within 10 seconds after injury, Trim72 mobilisation leads to formation of Mini-dysferlin$_{C72}$ and Trim72 lattices which help reseal the membrane with Annexin1 (Lek et al. 2013). As the aged post-ischaemic hearts show significant down-regulation of Cav3 (Chapter 3, Figure 3.5B), it is possible that the targeting of these Dysf and Trim72 patches to sites of injury may be affected by the decreased expression of Cav3. This remains to be shown in the ageing hearts and should be investigated using imaging techniques and/or immunocopreciptation.

Another member of the ferlin family, myoferlin, is up-regulated (mRNA) in response to damaged myotubes and in Dysf-deficient mice, with up-regulation of myoferlin thought to act as compensatory response (Davis et al. 2008; Demonbreun et al. 2010). In COS-7 cells, myoferlin participates in membrane repair that is dependent on *Cav1* expression as *Cav1* knockdown results in loss of this repair response (Bernatchez et al. 2009). In the same study, myoferlin was shown

to partially colocalise with another membrane-repair protein known as Dynamin-2 and also Cav1. The resulting protein complexes have been shown to be necessary for membrane-repair response in COS-7 cells (Bernatchez et al. 2009). In the ageing hearts used in this study, *Myof* showed significant up-regulation in 48-week old *vs.* 16 and 32-week old normoxic hearts whilst its expression in the post-ischaemic hearts did was not altered (Figure 7.5B). The exact role of myoferlin in cardioprotection is currently unknown.

7.3.4.1 Transcriptional analysis of calpain expression

In the normoxic and post-ischaemic ageing heart, *Capn1* (Figure 7.6A) and *Capn2* (Figure 7.6B) expression did not show any changes in the ageing. Minor down-regulation (1.3-fold) of *Capn2* in the 48-week old post-ischaemic vs age-matched normoxic hearts was detected (Figure 7.6B). Unlike *Capn2* and *Capn3*, effects of ageing were highly significant for *Capn3* as shown by two-way ANOVA analysis (Table 7.6). *Capn3* showed a significant 4-fold up-regulation in the 32 and 48-week old normoxic hearts when compared to younger hearts (Figure 7.6C). Similarly, this affect was also present in the post-ischaemic ageing hearts which showed 3-fold overexpression when compared to younger hearts (Figure 7.6C). Similar to *Capn2*, repression (1.6-fold) of *Capn3* in the 48-week old post-ischaemic vs age-matched normoxic hearts was observed (Figure 7.6C).

Table 7.6 Two-way analysis of variance of age and post-ischaemic effects on calpain transcript expression

Gene	Factor	Mean Square	F (DFn, DFd)	% of Total Variation	*P* value
Capn1	Age	0.507	1.38 (3,38)	8.4%	0.2647
	Ischaemia	1.599	4.34 (1,38)	8.8%	**0.0440**
	Age*Ischaemia	0.269	0.73 (3,38)	4.5%	0.5380
Capn2	Age	0.130	1.80 (3,40)	9.2%	0.1627
	Ischaemia	0.771	10.65 (1,40)	18.1%	**0.0023**
	Age*Ischaemia	0.064	0.88 (3,40)	4.5%	0.4574
Capn3	Age	11.42	49.05 (3,40)	73.3%	**< 0.0001**
	Ischaemia	2.174	9.34 (1,40)	4.7%	**0.0040**
	Age*Ischaemia	0.332	1.42 (3,40)	2.1%	0.2498

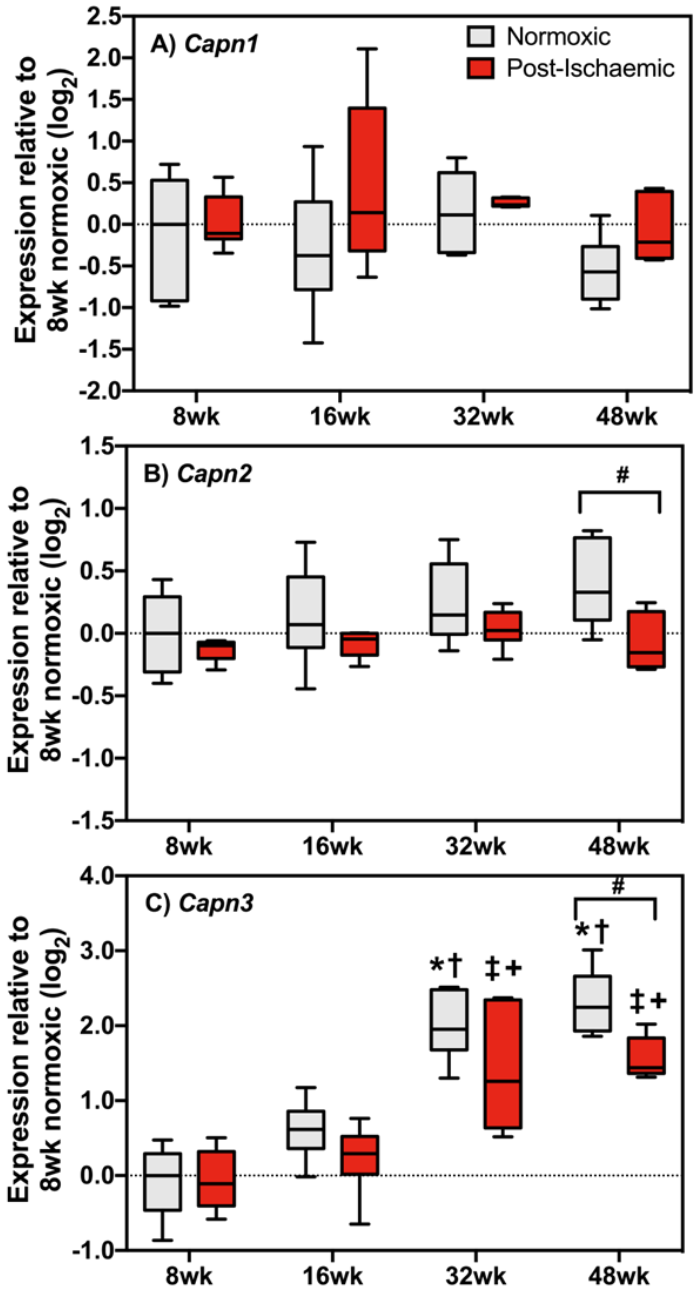


Figure 7.6 Age dependent expression of calpain transcripts in normoxic and post-ischaemic male hearts. Shown are boxplots detailing expression changes determined by RT-qPCR for **A)** *Capn1*, **B)** *Capn2* and **C)** *Capn3* in the normoxic (grey boxes) and post-ischaemic (red boxes) heart. Boxes indicate the interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values. * $P < 0.05$ vs 8-week old normoxic; † $P < 0.05$ vs 16-week old normoxic; ‡ $P < 0.05$ vs 8-week old post-ischaemic; + $P < 0.05$ vs 16-week old post-ischaemic. (n=6/group).

Calpains are cytosolic Ca^{2+}-activated cysteine proteases that are thought to be involved in variety of cardiovascular diseases such as heart failure, diabetes, and ischaemia-reperfusion (Letavernier et al. 2012). Fifteen calpain genes are encoded in the human genome, with the heart demonstrating high expression of Calpain1, 2, 3 and 4 (Sorimachi & Ono 2012; Sandmann, Yu & Unger 2001). I-R results in activation of several families of calpains which target numerous proteins in the cell including contractile proteins (i.e. troponin, vinculin) and membrane associated proteins (i.e. dystrophin) (Neuhof & Neuhof 2014; Müller, Hryshko, Dhalla & 2013). Calpains have been implicated in plasma membrane repair by cleaving Dysferlin into mini-dysferlin$_{C72}$ which forms patches with Trim72 at sites on injury (Lek et al. 2013). There were no changes in *Capn1* and *Capn2* expression in the ageing normoxic and post-ischaemic hearts (Figure 7.6A and 7.6B).

However, compared to their age-matched normoxic counterparts, the 48-week old post-ischaemic hearts showed significant down-regulation of *Capn2* and *Capn3* (Figure 7.6A-B). *Capn1* and *Capn2* have been suggested to be the essential calpains but not *Capn3* in the plasma membrane repair response of muscle fibres (Mellgreen et al. 2009). However, mutations and/or deficiency of *Capn3* and *Capn4* also have been shown to result in disruption of plasma membrane repair in skeletal and cardiomyocytes (Huang et al. 2008; Taneike et al. 2011). *Capn3* has been shown to cleave membrane-repair proteins such as Anxa1, Anxa2 and AHNAK which reduces their affinity to Dysferlin and may therefore disrupt Dysferlin-mediated repair (Lennon et al. 2003; Huang et al. 2008). Therefore, significant induction of *Capn3* in the post-ischaemic hearts (figure 7.11C) may impair repair mechanisms associated with Anxa1, Anxa2 and AHNAK. Besides its association with plasma membrane repair, Capn3 is associated with sarcomeric remodelling by cleaving myofibrillar proteins such as titin and vimentin (Guyon et al. 2003). Enhanced activity of calpains in the heart has been shown to result in sarcomeric disruption, myocardial stunning and ventricular remodelling after myocardial infarction (Galvez et al. 2007; Barta et al. 2005; Kudo-Sakamoto et al. 2014; Ma et al. 2012). However, it is of note that some Calpain3 activity is required for normal sarcomeric maintenance as Capn3-deficient mice show evidence of insoluble protein aggregates in skeletal muscles, similar to clinical observations in patients with *CAPN3* mutations (Kramerova et al. 2005; Hauerslev et al. 2012). Taken together, combined up-regulation of *Trim54*

and *Capn3* in the 32- and 48-week old hearts may result in increased degradation of sarcomeric contractile apparatus which may subsequently be responsible for decreased contractile efficiency observed in the 32- and 48-week old male hearts (Figure 3.2A).

7.3.5.1 Transcriptional analysis of Smpd expression

Smpd1 expression showed no changes in the normoxic ageing male heart (Figure 7.7A). Although there was a minor down-regulated trend of *Smpd1* in the 16 and 48-week old post-ischaemic heart hearts (Figure 7.7A) as further suggested by two-way ANOVA analysis (Table 7.7), post-hoc analysis showed this trend was not significant. Expression analysis of *Smpd2* revealed non-significant up-regulation in the 16 and 32-week old normoxic heart relative to 8-week old counterparts. This trend for *Smdp2* became significant in 16- and 32-week old post-ischaemic hearts (relative to 8-week old), whilst at the 48-week old time point its expression returned close to baseline (Figure 7.7B).

Table 7.7 Two-way analysis of variance of age and post-ischaemic effects on acid-spingomyolinase transcript expression

Gene	Factor	Mean Square	F (DFn, DFd)	% of Total Variation	*P* value
Smpd1	Age	0.186	4.23 (3,37)	20.1%	**0.0115**
	Ischaemia	0.494	11.23 (1,37)	17.8%	**0.0019**
	Age*Ischaemia	0.031	0.70 (3,37)	3.3%	0.5590
Smpd2	Age	1.789	9.14 (3,38)	38.1%	**0.0001**
	Ischaemia	0.280	1.43 (1,38)	2.0%	0.2391
	Age*Ischaemia	0.404	2.07 (3,38)	8.6%	0.1211

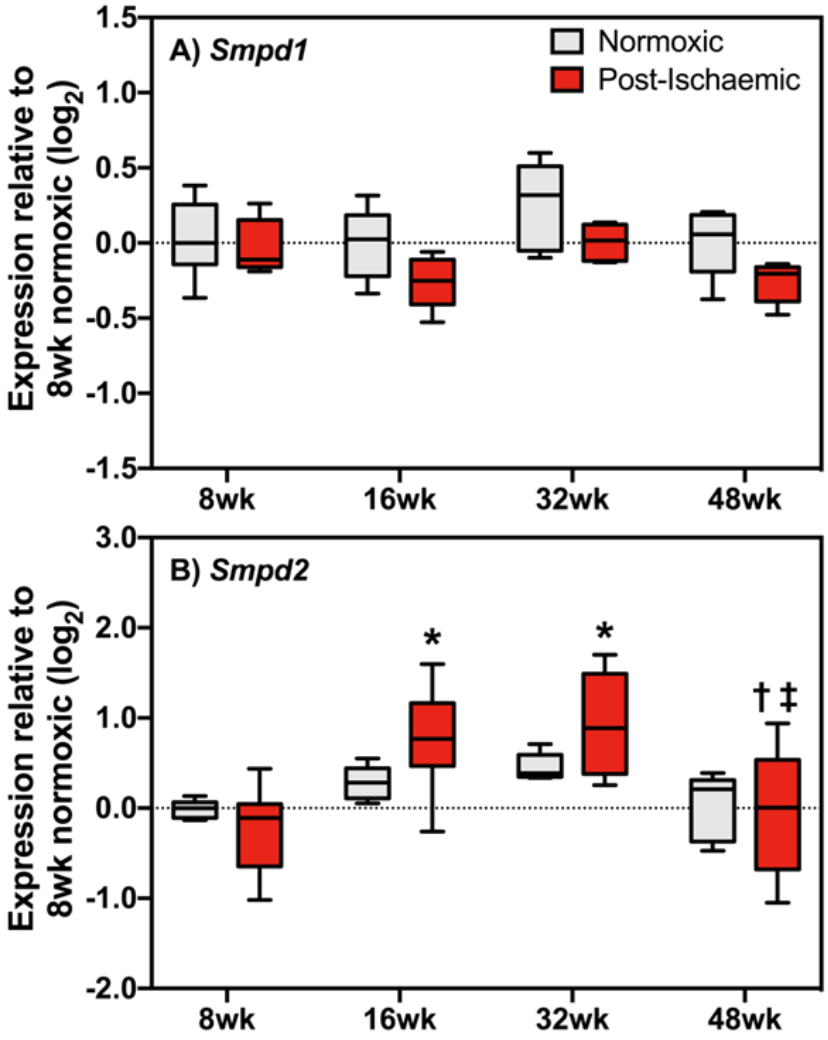


Figure 7.7 Age dependent expression of acid-spingomyolinase transcripts in normoxic and post-ischaemic male hearts. Shown are boxplots detailing expression changes determined by RT-qPCR for **A)** *Smpd1* and **B)** *Smpd2* in the normoxic (grey boxes) and post-ischaemic (red boxes) heart. Boxes indicate the interquartile range (25%-75%) with the horizontal bar within each box indicating the median. The whiskers show the minimum and maximum values. * $P < 0.05$ *vs.* 8-week old post-ischaemic; † $P < 0.05$ vs 16-week old post-ischaemic; ‡ $P < 0.05$ vs 32-week old post-ischaemic. (n=6/group).

The acid spingomyolinase (Smpd) family consists of four members comprising of *Smpd1*, *Smpd2*, *Smpd3* and *Smpd4*. The encoded proteins differing in optimum pH activity, cation dependence,

tissue distribution and subcellular distribution (Marchesini & Hannun 2004). Clinically, SMPD-deficiency due to *SMPD1* gene mutations results in the lysosomal storage-related neurodegenerative disease Niemann–Pick type (Schuchman & Wasserstein 2015). SMPDs participate in pathophysiology of cardiac and vascular dysfunction with various SMPD inhibitors suggested to be therapeutics for IHD (Pavoine et al. 2009). Smpds convert sphingomyelin, which are enriched in caveolae, into ceramide (Liu & Anderson 1995). Following I-R, ceramide formation is enriched in regions of Ca^{2+} influx, with preconditioning and pharmacological inhibition of Smpd shown to reduce I-R-induced cell death and decrease ceramide synthesis (Argaud et al. 2004). However, some expression of Smpd appears necessary as pharmacological inhibition of Smpd1 following membrane disruption results in impaired restoration of plasma membrane integrity (Tam et al. 2010). Indeed, delivery of recombinant human ASM to Niemann–Pick type A fibroblast has been shown to restore membrane integrity (Tam et al. 2010).

Smpds can influence the degree of Cav3-Cavin1 co-localisation as delivery of Smpd has been shown to result in significant increase of this co-localisation when compared to mock controls (Corette et al. 2013). The exact mechanism of this interaction is currently unknown, however, such enhanced co-localisation would assist in plasma membrane repair mechanisms and both Cav3 and Cavin1 are crucial to this process by mechanisms discussed previously (Corette et al. 2013; Zhu et al. 2011). In the ageing post-ischaemic hearts, *Smpd1* did not show significant changes with ageing although a minor trend was present in the 48-week old hearts. However, its activity as a plasma membrane repair protein may be compromised as Smpd1-mediated repair has been shown to be reliant on Cav3 and possibly Cavin1, which as discussed previously in Chapter 3 are down-regulated in the middle-aged hearts (Defour et al. 2014; Corrette et al. 2013).

7.4 CONCLUSION

In this study, we investigated the expression of a variety of membrane repair-related transcripts in the ageing male normoxic and post-ischaemic hearts, which were largely uncharacterised in the ageing heart. As previously discussed, membrane repair mechanisms have been shown to be crucial mediators of cardioprotective responses such as IPC and IPOST, as deficiency of a plasma membrane repair protein, Trim72, results in a loss of cardioprotection (Cao et al. 2010; Zhang et al. 2011). Caveolae and its coat-proteins have been implicated in the membrane repair response as knockdown of *Cav3*, *Cav1* and *Cavin1* results in ablated membrane-repair response (Corrotte et al. 2013; Zhu et al. 2011). Indeed, preservation of the sarcolemmal structures and membrane integrity as shown by decreased Troponin leakage following I-R are features of the Cav3-overexpression and IPC (Tsutsumi et al. 2008; Murry, Jennings & Reimer 1986). Involvement of caveolae was noted as early as 1981 in Duchenne muscular dystrophy samples as noted by significant increase in the number of caveolae seen under EM in these samples (Bonilla, Fischbeck & Schotland 1981). It has been proposed that formation of caveolae at the site of injury which combine with caveolae-like vesicle to internalise and isolate the damaged site leading to repair (Corrette et al. 2013).

To further highlight to role caveolae and its coat proteins, mutation in *CAV3* and *CAVİN1* result in several forms of muscular dystrophies, which affect sarcolemma membrane stability and disorganization of skeletal muscle T-tubule network (Gazzero et al. 2010; Hayashi et al. 2009). Expression for most of the plasma membrane-repair transcripts investigated in this study were unaffected by ageing, most notably *Trim72*, *Capn1* and *Anxa6*. Although *Anxa5* and *Smpd1* showed minor down-regulated trends in the 48-week old post-ischaemic hearts, whilst being non-significant, the loss of Cav3 and Cavin1 in the ageing post-ischaemic hearts (Chapter 3) may disrupt their roles in repair response (I.e. for Trim72) as they have been shown to be reliant on caveolae and their coat proteins for targeting to disruption sites (Cao et al. 2010; Zhu et al. 2011). For example, Cav3-deficiency disrupts the targeting of dystrophin-glycoprotein complex, associated with plasma membrane stability, to caveolae in conjunction with the loss of α-sarcoglycan expression (Woodman et al. 2002).

As shown in Chapter 3, the ageing heart displays reduced contraction efficiency following I-R. In addition to membrane repair mechanisms, we investigated transcripts related to sarcomeric

remodelling which certain members of the membrane repair related transcripts shown to also participate in this process. Degradation of contractile-related proteins is a normal physiological process whereby damaged and misfolded proteins are removed by the ubiquitin proteasome system in the heart (Willis et al. 2009). As shown in Figure 3.2A, the 32- and 48-week old male hearts show reduced recovery following I-R which may be due to enhanced degradation of contractile apparatus from proteases, (i.e. Calpains), increased protein turnover (via Trim52) or reduced efficacy or repair mechanisms (i.e. Annexins). Members of Calpains, Trim, Annexins and Dysferlins have been shown to localise in sarcomeric apparatus such as T-tubules in muscle fibres and are thought to maintain the integrity of the T-tubule network (Waddell et al. 2011; Voigt et al. 2013). As previously discussed, Capn3 and Trim54 are associated with sarcomeric remodelling by cleaving myofibrillar proteins and ubiquitin-mediated muscle protein turnover respectively (Guyon et al. 2003; Perera, Mankoo & Gautel 2012). Significant-up regulation of *Trim54* was observed in both the normoxic and post-ischaemic ageing hearts. As a result of significant induction of *Capn3* and *Trim54* in the 32- and 48-week old post-ischaemic hearts, enhanced degradation of sarcomeric proteins may occur as overexpression of *Trim54* has been shown to result in loss in expression of FHL2 and γ-filamin (Fielitz et al. 2007). However, this remains to be shown.

Recently, Poloxemer 188, a biocompatible polymer has been proposed as a novel therapeutic for patients with muscular dystrophies (i.e. Duchenne) and has been shown to protect against ischaemic injury in skeletal muscle (Murphy et al. 2010), neurons (Colbassani et al. 1989), heart failure (Juneman et al. 2012) and various types of cellular of injuries in other tissues (see review Moloughney & Weisleder 2012). Future studies, using kinetic measurements, should investigate whether the middle-aged and aged hearts show attenuated repair mechanisms when compared to young/mature counterparts. Immunoblot analysis should be performed on Anxa5 and Smpd1 for comparisons in the 8- and 48-week old hearts as a trend in decrease was detected in these hearts. In addition, the substrates of *Capn3* and *Trim54* should be investigated whether enhanced degradation/cleavage occurs in the aged hearts as a result of increased expression of these transcripts. Future studies should also investigate the expression of notable transcripts crucial for membrane-repair including members of EHD, dynamin, dystrophin complex.

8. Chapter Eight

Conclusion

8.1 FINAL CONCLUSION

Ischaemic heart disease is the most prevalent CVD (44%) and the largest single leading cause of death in Australia with over 19,000 reported deaths in 2011 (ABS 2011; Heart Foundation 2012). The incidence of IHD increases with age, affecting 29% of those aged 75 years and over and 47% of those aged 85 years and over. Timely reperfusion of the ischaemic myocardium can result in preservation of over 50% of the myocardial tissue (Schömig, Ndrepepa & Kastrati 2006). However, reperfusion is a 'double-edged sword' as restoration of blood flow may contribute to as much as 50% of the final infarct size (Jennings et al. 1960). Cardioprotection are interventions designed to prevent or reduce injuries (oncosis, arrhythmias, contractile dysfunction) from myocardial infarction, especially those caused by I-R (Heusch 2013). Experimentally, cardioprotection can be exploited via ischaemic conditioning methods (e.g. IPC and IPOST) or pharmacologically (e.g. A_1AR and δ-opioid receptor activation). Although these interventions are successful in reducing infarct size and post-ischaemic dysfunction in young hearts, there is an age-related impairment of these methods in the aged hearts. Both IPC and pharmacological agonism have been shown to be reliant on the presence of caveolae, which house a variety of cardioprotective receptors as well as their signalling subunits (Tsutsumi et al. 2008; Peart et al. 2014).

As we and others have shown, concurrent with the reduction of ischaemic tolerance in the aged hearts is the age-related decline of Cav3 protein expression, which is critical for cardiomyocyte caveolae formation (Chapter 3; Kidd et al. 2010; Peart et al. 2014). To further support the role of caveolae in modifying ischaemic tolerance in the aged heart, restoration of caveolae in the aged heart results in enhanced recovery of these hearts (Kidd et al. 2010). Conversely, depletion of caveolae via MβCD and *Cav3*-deficiency results in reduced ischaemic tolerance (Chapter 5, See Hoe et al. 2014). In addition to the reduction of *Cav3*, here we show that there is also age-related loss of *Cavin1* and *Popdc1* which have recently also been shown to be crucial determinant of cardiomyocyte caveolae and may also enhance the loss of caveolae in these hearts. Surprisingly, the female ageing myocardium showed similar mRNA expression patterns of *Cav3*, *Cavin1* and *Popdc1* especially in the post-ischaemic hearts. Furthermore, the age-related reduction of post-ischaemic Cav3 and cavin1 protein in the middle-aged female hearts was also similar to their male

counterparts. Taken together, age-related decrease in caveolae forming transcripts may be responsible for reduction of caveolae in the male and female middle-aged heart as we and others show (Peart et al. 2014). Consequently, the reduction in caveolae may underpin the middle-aged hearts sensitisation to ischaemic insult and desensitisation to cardioprotective therapies. We then investigated whether the reduction of *Cav3* and *cavin1* may be due to miRNA-mediated repression of these transcripts. The age-related decrease of *Cav3* and *cavin1* did not show inverse-correlation with candidate miRNAs predicted to target these transcripts although it remains a possibility that other miRNAs not used in this study may be control their expression. Of the miRNAs assessed, the induction of *miR-15b*, *miR-22* and *miR-92a* in the middle-aged hearts was consistent with their respective roles in the cell and may contribute loss of cardioprotection in these hearts. Lastly, we investigated the expression of several plasma membrane repair transcripts, which remain largely uncharacterised in the ageing heart.

Membrane-repair mechanisms are crucial mediators of cardioprotective mechanisms such as IPC and IPOST (Zhang et al. 2011; Cao et al. 2010). Furthermore, members of plasma membrane repair machinery such as Trim72 require presence of Cav3 and Cavin1 in order to be targeted to sites of injury. Of the plasma membrane repair transcripts, we did not detect significant differences in the post-ischaemic ageing hearts although *Anxa5* and *Smpd1* showed a minor trend in down-regulation in the middle-aged post-ischaemic hearts. Although the expression of *Trim72*, *Anxa5* and *Smpd1* did not show significant changes in the post-ischaemic heart, their targeting to sites of injury may be affected by the decreased Cav3 and Cavin1 expression although this remains a hypothesis. We also investigated the expression of *Trim54* and calpain transcripts which are implicated in the maintenance of the sarcomere. In the 32- and 48-week old post ischaemic hearts there was a notable induction of *Trim54* and *Capn3*, and may be partially responsible for reduced recovery in the 32- and 48-week old hearts although it remains to be shown their substrates are affected by this induction.

Key findings in of this study;

- Loss of endogenous cardioprotection is evident in the 48-week old male (middle-aged) hearts as shown by enhanced LDH release (Figure 3.2B). Although LDH release was not significant in the 32-week old heart, there is evidence of post-ischaemic dysfunction as shown by reduced pressure developed pressure in these hearts similar to that of 48-week old hearts (Figure 3.2A).
- *Cav3* mRNA expression is down-regulated in the normoxic hearts as early as 16-week old hearts (Figure 3.4C) with the 48-week old hearts shown to display significant down-regulation at the proteomic level (Figure 3.5A). Similarly, the 32 and 48-week old post-ischaemic hearts showed down-regulation of *Cav3* transcript (Figure 3.4C), with significant Cav3 protein down-regulation observed in the 48-week old heart (Figure 3.5B). The reduction in Cav3 protein in the 48-week old heart correlated with the significant reduction of ischaemic tolerance in these hearts.
- Transcriptional analysis showed significant *Cavin1* down-regulation in the normoxic 48-week old male hearts (Figure 3.6A) although this was borderline non-significant at the proteomic level (Figure 3.7A). The post-ischaemic heart also showed significant *Cavin1* down-regulation in the 48-week old heart as shown by RT-qPCR (Figure 3.6A) and immunoblotting (Figure 3.7B). The reduction in Cavin1 protein in the 48-week old heart correlated with the significant reduction of ischaemic tolerance in these hearts.
- Although *Popdc1* and *Popdc3* did not show significant changes in the normoxic ageing male hearts, there was an age-related repression these transcripts in the post-ischaemic male hearts. More specifically, *Popdc1* mRNA showed significant down-regulation in the 32 and 48-week old hearts whilst *Popdc3* showed trend towards significant down-regulation (P=0.15) in the 48-week old hearts (Figure 3.8A and 3.8C). Notably, there was also reduction of these transcripts in the 32 and 48-week old post-ischaemic hearts when compared to their age-matched normoxic counterparts (Figure 3.8A and 3.8C).
- Post-ischaemic female hearts showed significant reduction in recovery in the 32 and 48-week old hearts similar to that of male counterparts (Figure 4.1A). Reduced recovery observed in the 32-week old hearts did not match the LDH release which also did not show

significant changes when compared to younger phenotypes. Significant LDH release was observed in the 48-week old female myocardium when compared to 8 and 16-week old counterparts (Figure 4.1B). Furthermore, we observed that contrary to other authors, the 48-week old female hearts displayed marked LDH release when compared to their age-matched male counterparts (Figure 4.1C). The menopausal state of the 48-week old hearts used in this study is unknown and future investigations should address this.

- Although transcriptional analysis did not suggest significant down-regulation of *Cav3* in female 48-week old normoxic hearts (Figure 4.3C), immunoblotting showed significant down-regulation of Cav3 (Figure 4.4A). Conversely, although transcriptional analysis showed significant Cav3 reduction in the ageing post-ischaemic female heart (Figure 4.3C), Cav3 down-regulation was borderline non-significant at the proteomic level (Figure 4.4B). Altogether the age-related down-regulation of *Cav3* appears present in the middle-aged female post-ischaemic heart similar to that of male counterparts.
- Transcriptional analysis and immunoblotting of Cavin1 in the female normoxic ageing hearts revealed no significant changes (Figure 4.5A & Figure 4.6A). However, although the post-ischaemic suggested no age-related transcriptional changes (Figure 4.5A), significant down-regulation of Cavin1 protein was observed in the 48-week old hearts (Figure 4.6B). The combined reduction of Cav3 and Cavin1 protein in the 48-week old post-ischaemic hearts coincided with the loss of I-R tolerance in these hearts.
- Of the three Popdcs mRNAs, only *Popdc3* showed significant age-related changes in the female myocardium. More specifically, *Popdc3* showed significant induction in the 32- and 48-week old post-ischaemic hearts when compared to age-matched normoxic counterparts (Figure 4.7C).
- Of the miRNAs assessed, only *miR-378* was consistently down-regulated in the normoxic and post-ischaemic heart (Figure 6.4E) although it remains to be shown whether its targets *Igf1r* and *Grb2* are down-regulated in the ageing hearts used in this study.
- Relative to their normoxic age-matched counterparts, we report up-regulation of I-R-related miRNAs: *miR-15*, *miR-20a*, *miR-24* and *miR-92a* in the post-ischaemic 48-week old heart (Figure 6.3), consistent with literature reports and their roles as pro-apoptotic

miRNAs. Similarly, for the ageing/senescence related miRNAs, we observed significant up-regulation of *miR-22* (Figure 6.4A) in the 48-week old post-ischaemic hearts vs age-matched normoxic, consistent with its role in cardiac ageing.

- There was no inverse correlation in expression detected for miRNAs targeting *Cav3* and *Cavin1* used in this study.
- After extensive optimisation of several crucial parameters for transfection, a modest degree transfection was achieved using *Cav3* miRNA vectors, comparable to that of the literature. However, this degree of transfection has been suggested to produce unreliable knockdown and was abandoned in lieu of pharmacological approaches.
- To show caveolae involvement in modifying ischaemic tolerance, pharmacological approach using cholesterol depletion (via MβCD) of cellular rafts was investigated. MβCD treatment following sI-R enhanced cell death compared to non-treated sI-R cells as shown by LDH release (Figure 5.16A) and Annexin V-PE and 7-AAD staining (Figure 5.16B).
- Of the membrane-related transcripts, only *Anxa5* showed significant down-regulation with ageing in the normoxic 16-, 32- and 48-week old hearts (Figure 7.4B). Whilst a minor down-regulated trend for *Anxa5* and *Smpd1* was observed in the 48-week old post-ischaemic hearts (vs 8-week old) which should be confirmed via immunoblotting.
- Protease transcripts *Trim54* and *Calpain3* showed significant up-regulation in the 32- and 48-week old post-ischaemic male hearts (Figure 7.2B and Figure 7.6C, respectively), which may contribute to post-ischaemic contractile dysfunction in these hearts by increased degradation of contractile proteins. However, this remains to be shown.

8.2 LIMITATIONS

As access and availability of human ventricular samples are rare, we were unable to compare between murine and human tissue models as to whether the age-related loss of *Cav3*, *Cavin1* and *Popdc1* also occurs in the ageing human hearts. The reduction in morphological caveolae should be confirmed using electron microscopy especially in the female hearts as this has not been shown in the female hearts whilst morphological reduction in caveolae has been confirmed in male hearts by other investigators (Peart et al. 2014). Although Cav3 and Cavin1 has been shown to be crucial for plasma membrane repair *in vitro*, it remains to be demonstrated whether the reduction of Cav3 and Cavin1 in the middle-aged hearts is sufficient enough to reduce the efficacy of plasma membrane repair mechanisms in middle-aged hearts used in this study. Although *Popdc1* showed significant transcriptional down-regulation in the ageing post-ischaemic male hearts, it remains to be shown whether this repression results in significant proteomic reduction. Significant up-regulation of *miR-15b*, *miR-20a*, *miR-22*, *miR-24* and *miR-92a* were observed in the 48-week old post-ischaemic myocardium relative to their age-matched normoxic counterparts. This may contribute to loss of ischaemic tolerance in these hearts by mechanisms detailed in Chapter 6. However, it remains to be shown whether their targets are significantly affected by this change in expression. Similarly, whilst significant repression of *miR-378* was observed in the 32- and 48-week old normoxic and post-ischaemic male hearts, it remains to be shown whether its mRNA targets are also impacted. Due to lack of concise indicators and lack of literature availability in menopausal state of the mice hearts, we are unaware of the progression of menopause and whether it even occurred in the 32- and 48-week old hearts used in this study. It is noteworthy that other studies may have avoided this uncertainty by the use the aged (74-week old) phenotype which is labelled as post-menopausal. Although we were unable to achieve high (>70%) transfection efficiencies using chemical transfection, the use of AAV vectors and or the recently released Lipofectamine 3000 may have resulted in greater transfection rates suitable for functional studies (sI-R etc.). Although we detected an increase in *Capn3* and *Trim54*, it remains to be shown whether this age-related induction of these transcripts result in increased degradation to their substrates.

8.3 FUTURE DIRECTIONS

The down-regulation of *Popdc1* should be confirmed via immunoblotting in the aged male and female hearts. Similarly, immunoblotting of Anxa5 and Smpd1 should also be employed as a small trend in decrease was observed for these transcripts in the 48-week old post-ischaemic vs 8-week old hearts. Although we investigated the expression of *Cav1* mRNA in the ageing heart, future studies should also look at phospho-Cav1 as phosphorylation of caveolins appear important for attachment of caveolae to the plasma membrane (Sowa et al. 2003). Furthermore, phosphorylation of caveolins has also been implicated in internalisation of caveolae which may relay cardioprotective signalling downstream. Therefore, a reduction in phospho-Cav1 may also explain reduced caveolae-mediated signalling in these hearts as phospho-Cav1 has been shown to be required for anaesthetic preconditioning (Patel et al. 2007). Other post-translational modification observed for caveolins is nitroysylation, which as mentioned previously plays a crucial role in cardioprotection, acylation of Cav1 which is has been shown to bind Cav1 to G-protein $G_{i1\alpha}$ in caveolae, and palmitoylation of Cav1, required for coupling Cav1 to the c-Src tyrosine kinase (Bakhshi et al. 2010; Galbiati et al. 1999; Lee et al 2001). Measurement of circulating estrogen in the ageing female heart may also be of interest and whether this correlates with the reduction of ischaemic tolerance in the 48-week old hearts. Future investigations should also replicate the male plasma membrane study on females in a similar fashion to Chapter 3 and 4.

The mRNA targets of *miR15b*, *miR-20a*, *miR-22*, *miR-24*, *miR-92a* and *miR-378* should be investigated in the normoxic and post-ischaemic ageing hearts which should be inversely-correlated with their expression. Additionally, >74 week-old mice (aged) should be used in further miRNA characterization to observe whether the senescence-related miRNAs such as *miR-34a* and *miR-34c* show differential expression at this time point as shown by others (Boon et al. 2013). Although there is plethora of literature available regarding the efficacy of canonical cardioprotective signalling, the efficacy of the plasma membrane repair mechanism in the aged heart remains largely uncharacterised. The association of plasma membrane repair protein Trim72 with Cav3 and Cavin1 should be compared in the young hearts vs. aged hearts whether the age-related reduction Cav3 and Cavin1 affects co-localisation of these proteins in the normoxic and post-ischaemic hearts. Methods to investigate this association could be co-

immunoprecipitation and/or confocal microscopy. Using immunoblotting, the substrates of Trim54 (i.e. FHL2 and γ-filamin) and Calpain3 (i.e. titin and vimentin) should also be investigated whether increased degradation of their substrates is present in the 32- and/or 48-week old post-ischaemic hearts used in this study (Fielitz et al. 2007). Overexpression of *Capn3* and *Trim54* could also help elucidate whether enhanced levels of these transcripts could result in increased cleavage their substrates. This may help partially explain the reduced recovery following I-R in the middle-aged hearts. The expression of other plasma membrane repair proteins such as dynamin1, dynamin2 and S100A11 should also be investigated. The expression of plasma membrane repair-related transcripts should also be investigated in female hearts whether there are any sex-related differences. The expression of caveolar bulb proteins such as EHD and Pacsin family of proteins should also be investigated as they have been shown to influence caveolae formation and morphology (Moren et al. 2012). The use of high-throughput technologies such as next-generation sequence or microarrays could be broadly used to investigate the expression of miRNA targets that showed significant changes in expression simultaneously also could be used to view regulatory networks affected by ageing i.e. plasma membrane repair mechanisms. The application of these technologies on the ageing female hearts used in this study would also help further elucidate sex-related differences. Although transgenic models have clearly shown Cav3 to be crucial determinant of cardioprotective signalling, currently no transgenic studies have investigated cavin1 terms of cardioprotection and should be investigated using deficiency and/or overexpression models (Tsutsumi et al. 2008; See Hoe et al. 2014).

9. Chapter Nine

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